

**MODULATION OF KIR6.1 CHANNELS HETEROLOGOUSLY
EXPRESSED IN HEK-293 CELLS
BY NICOTINE AND ACETYLCHOLINE**

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By

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ABSTRACT

ATP-sensitive K^+ channels (K_{ATP}) channels were first described in the cardiac muscles. K_{ATP} channels are a complex of regulatory sulphonylurea receptor subunits and pore-forming inward rectifier subunits such as Kir6.1. Nicotine, an exogenous substance, adversely affects cardiovascular function in humans. Acetylcholine (ACh) is well known as a key neurotransmitter of the parasympathetic nervous system. ACh effects are usually related to binding to muscarinic receptors and stimulating second messengers that relay and direct the extracellular signals to different intracellular destinations, resulting in modulated cellular activity. We hypothesize that nicotine and ACh may modulate Kir6.1 channels via different mechanisms. Using the whole cell patch-clamp technique, the interactions of nicotine and ACh with Kir6.1 subunit permanently expressed in Human Embryonic Kidney (HEK-293) cells as well as the underlying mechanisms were studied.

Non-transfected HEK-293 cells possess an endogenous K^+ current with current density of -3.2 ± 1.4 pA/pF at -150 mV ($n = 9$). Stable expression of Kir6.1 subunits cloned from rat mesenteric artery in HEK-293 cells yielded a detectable inward rectifier K_{ATP} current (-23.9 ± 1.6 pA/pF at -150 mV, $n = 6$). In the presence of 0.3 mM ATP in the pipette solution, nicotine at 30 and 100 μ M increased the expressed Kir6.1 currents by 42 ± 11.8 and $26.2 \pm 14.6\%$, respectively ($n = 4-6$, $p < 0.05$). In contrast, nicotine at $1-3$ mM inhibited Kir6.1 currents ($p < 0.05$). Nicotine at 100 μ M increased the production of superoxide anion ($O_2^{\cdot -}$) by $20.3 \pm 5.7\%$ whereas at 1 mM it significantly decreased the production of $O_2^{\cdot -}$ by $37.7 \pm 4.3\%$. The hypoxanthine/xanthine oxidase (HX/XO) reaction was used as a source of $O_2^{\cdot -}$. Co-application of HX and XO to the transfected

HEK-293 cells resulted in a significant and reproducible increase in Kir6.1 currents. Tempol, a scavenger of O_2^- , abolished the stimulatory effect of HX/XO on Kir6.1 currents. Tempol also abolished the stimulatory effect of 30 μ M nicotine on Kir6.1 currents (-28.3 ± 6.1 pA/pF vs. -31.2 ± 7.3 pA/pF at -150 mV, $n = 6-9$ for each group, $p > 0.05$).

In the presence of 0.3 mM ATP in the pipette solution, ACh concentration-dependently increased the expressed Kir6.1 currents. At 1 μ M, ACh increased Kir6.1 currents from -19 ± 2.5 to -31.7 ± 2.1 pA/pF ($n = 8$, $p < 0.05$). Pretreatment of the transfected HEK-293 cells with either 2 or 20 μ M atropine, 100 nM α -bungarotoxin, 100 μ M mecamylamine, 2 μ M prazosin, 1 μ M propranolol, or 10 μ M dihydro- β -erythroidine hydrobromide did not alter the stimulatory effect of ACh on Kir6.1 currents ($n = 4 - 5$ for each group, $p < 0.05$). When intracellular ATP was increased to 5 mM, ACh at 10 μ M still exhibited its stimulatory effect (-16.4 ± 2.3 to -25.5 ± 3.8 pA/pF, $n = 8$, $p < 0.05$). For the first time, the present study provides an insight for the interactions of nicotine and ACh with Kir6.1 subunits. Our data demonstrate that micromolar concentration of nicotine and ACh stimulated Kir6.1 channels. Nicotine at millimolar concentrations inhibited Kir6.1 channels. The dual effect of nicotine, not mediated by nAChR, are mediated partially by O_2^- levels in the cells. The ACh excitatory effect is mediated neither by an AChR-dependent mechanism, nor by alteration in ATP metabolism. This study challenges the traditional explanations for the receptor-mediated effects of nicotine and ACh on ion channels and opens a new door to understand the effects of nicotine and ACh on K_{ATP} channels in many cellular systems.

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DEDICATED TO OUR BELOVED SONS
KAIS AND FADI

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LIST OF ABBREVIATIONS

α -BTX	α -bungarotoxin
2K-1C rats	two-kidney-one clip rats
5-HD	5-hydroxydecanoic acid
a.m.b.	after membrane brake
AC	adenylyl cyclase
AChE	acetylcholinesterase
ADP	adenosine diphosphate
AMP-PCP	adenylylmethylenediphosphate
AMP-PNP	adenylyl imidodiphosphate
AP	action potential
ATP	adenosine triphosphate
AV block	atrioventricular block
Ca ²⁺ channels	calcium channels
cGMP	cyclic guanosine monophosphate
ChAT	choline acetyltransferase
D β E	dihydro- β -erythroidine hydrobromide
DMPP	1, 1-dimethyl-4-phenylpiperazinium iodide β
ECG	electrocardiogram
EDRF	endothelium-derived relaxing factor
EGTA	ethylene glycol-bis(beta-aminoethyl ether)- N,N,N',N'-tetraacetic acid
FBS	fetal bovine serum

G-F-G	glycine-phenylalanine-glycine
GSH-Px	glutathione peroxidase
GSSG	Oxidized glutathione
H ₂ O ₂	hydrogen peroxide
HEK-293	human embryonic kidney cell line
HOCl	hypochlorous acid
HOO [·]	hydroxyperoxyl radicals
HX	hypoxanthine
I _f	pacemaker current (funny current)
IbTX	iberiotoxin (big conductance K _{Ca} channel blocker)
IC ₅₀	the concentration of a drug that is required for 50% inhibition of the maximum effect
I _{GJ}	gap junctional current
I _{k,ACh}	ACh-sensitive K ⁺ current
I _{K,ACh} channel	acetylcholine-activated potassium current
I _{to}	transient outward K ⁺ channel currents
K _{ATP} channels	ATP-sensitive K ⁺ channels
K _{Ca}	Ca ²⁺ dependent K ⁺ channels
<i>Kcnj11</i>	potassium inwardly-rectifying channel, subfamily J, member 11
<i>Kcnj8</i>	potassium inwardly-rectifying channel, subfamily J, member 8
KCO	K ⁺ channel openers
Kir6.1 Ab	anti-Kir6.1 antibody

Kv	voltage dependent K ⁺ channels
L-type Ca ²⁺ channels	long-lasting type of Ca ²⁺ channels
mAChR	muscarinic acetylcholine receptors
Na ⁺ channels	sodium channels
nAChR	nicotinic acetylcholine receptors
NAD ⁺	nicotinamide adenine dinucleotide
NBD1	nucleotide binding domain 1
NBD2	nucleotide binding domain 2
NIDDM	non-insulin dependent diabetes mellitus
NO	nitric oxide
O ₂ ^{·-}	superoxide anion
OH [·]	Hydroxyl radical
ONOO [·]	peroxynitrite
OOR [·]	peroxyl radicals
OR [·]	Alkoxy radicals
PDE	phosphodiesterase
PGI ₂	prostacyclin
PKA	cAMP-dependent protein kinase.
PKG	cGMP-dependent protein kinase
PNU-37883A	4-morpholinecarboxamidine-N-1-adamantyl-N ⁻ -cyclohexyl-hydrochloride
PSS	physiological saline solution
RO [·]	alkoxy radical
ROO [·]	peroxyl radical

ROS	reactive oxygen species
SCD	sudden cardiac death
SMCs	smooth muscle cells
SOD	superoxide dismutase
SUR	sulphonylurea receptor
Tempol	4-hydroxy-2,2,6,6-tetramethyl-piperidine-1 oxyl
TMD	transmembrane domain
UDP	nucleoside diphosphate
VACHT	vesicular ACh transporter
XO	xanthine oxidase

LIST OF CHEMICALS

The following chemicals were obtained as shown in the list below:

(-)-Nicotine [(-)-1-Methyl-2-[3-pyridyl] pyrrolidine]			Sigma
α -BTX			Sigma
DMPP			Sigma
Acetylcholine			Sigma
Anti-Kir6.1 Ab		antibody prepared in our laboratory (Sun et al., 2004)	
ATP			Sigma
Atropine			Sigma
CaCl ₂			Sigma
Cytisine			Sigma
D β E			Sigma
EGTA			Sigma
Genticine			GIBCO
Glucose			Sigma
HEPES			Sigma
Hypoxanthine (HX)			Sigma
IbTX			Sigma
KCl			Sigma
Mecamylamine			Sigma
MgCl ₂			Sigma
Na ₂ -ATP			Sigma

NaCl	Sigma
pCR2.1-rvKir6.1 cDNA	prepared in our laboratory (Cao et al., 2002)
PNU-37883A	gift from Pfizer Canada
Prazosin	Sigma
Propranolol	Sigma
Tempol	Sigma
Xanthine oxidase (XO)	Sigma Chemical, St.Louis, MO.

1. GENERAL INTRODUCTION

1.1. K_{ATP} CHANNELS

ATP-sensitive K^+ channels (K_{ATP} channels) were first described in the cardiac muscle by Noma (1983) and were characterized by inhibition when the ATP concentration at the cytoplasmic surface was increased to millimolar levels (Ammala et al., 1996a). Subsequently, K_{ATP} channels were found in various tissues, including pancreatic β -cells, skeletal and smooth muscle, pituitary, and brain. K_{ATP} channels couple the cell's metabolic status to its electrical activity and play an important role in various cellular functions as sensors of intracellular ATP and ADP (Fig. 1.1) (Seino and Miki, 2003). In pancreatic β -cells, the increase in ATP/ADP ratio generated by glucose stimulation closes the K_{ATP} channels to elicit the secretion of insulin, the primary hormone of glucose homeostasis (Ammala et al., 1996b). Thus K_{ATP} channels are crucial in the regulation of glucose-induced insulin secretion. They also are the targets for sulphonylureas, insulin secretagogues widely used as oral hypoglycemic agents in the treatment of non-insulin-dependent diabetes (NIDDM). K_{ATP} channels in cardiac and skeletal muscle have been implicated in cell excitability, cytoprotection, and the cellular loss of K^+ during ischemia, hypoxia, or other metabolic insults (Ammala et al., 1996a). In brain, K_{ATP} channels are involved in neuronal excitability and appetite control, and in cytoprotection in ischemia and hypoxia. K_{ATP} channels also have been implicated in other functions, including smooth muscle relaxation and vasodilatation, secretion of pituitary hormone, K^+ recycling in renal epithelia, and oocyte maturation (Nelson and Quayle, 1995).

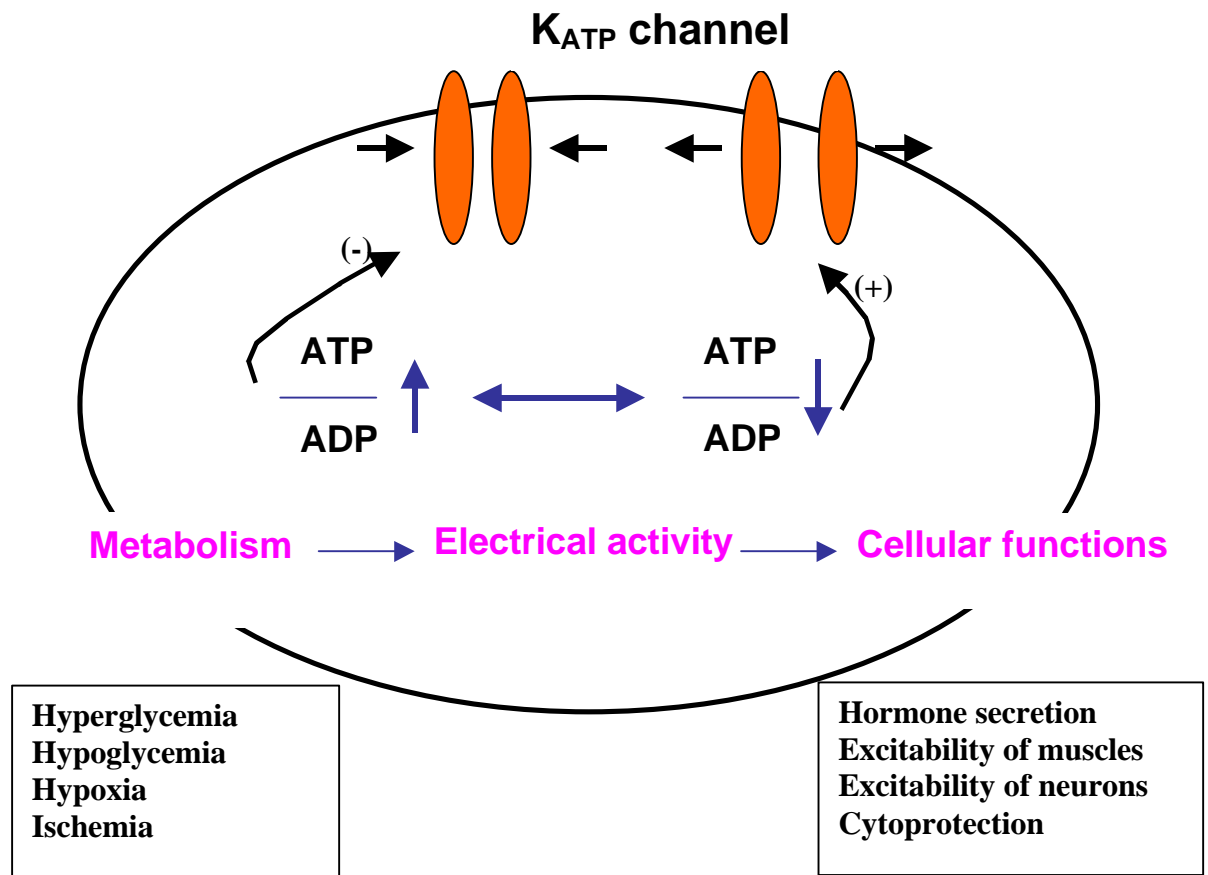


Figure 1.1. K_{ATP} channel as metabolic sensor. K_{ATP} channels play an important role in the cellular responses of various tissues to altered metabolic states, including hyperglycemia, hypoglycemia, ischemia, and hypoxia.

Electrophysiological studies have shown that the kinetics and pharmacological properties of K_{ATP} channels vary among different tissues, suggesting structurally and functionally distinct types (Cao et al., 2002; Ammala et al., 1996b). K_{ATP} channels are a complex of the Kir6.x subunit, a member of the inwardly rectifying K^+ channel family, and the sulphonylurea receptor (SUR) subunit, a member of the ATP-binding cassette (ABC) superfamily (Cao et al., 2002). Various combinations of the Kir6.x subunit with SUR subunits have been shown to reconstitute the functionally diverse K_{ATP} channels.

1.2. PROPERTIES OF K_{ATP} CHANNELS IN NATIVE TISSUES

1.2.1. Biophysical properties of K_{ATP} channels

K_{ATP} channels are highly selective for K^+ and Rb^+ and have a relative permeability to Na^+ over K^+ (P_{Na}/P_K) of ~ 0.01 . The current-voltage relationships of K_{ATP} channels show the property of weak inward rectification, the outward current being smaller than the inward current (Inagaki et al., 1995). The inward rectification of K_{ATP} channels is due to a voltage-dependent block of outward currents by internal cations such as Mg^{2+} and Na^+ . Single channel conductance measured in the inside-out configuration ranges from ~ 50 to 75 pS in pancreatic β -cells and ~ 70 to 90 pS in cardiac cells for inward currents under symmetrical K^+ conditions of 140 mM on both sides of the membrane (Inagaki et al., 1996). A fundamental characteristic of K_{ATP} channel openings is that they are time-independent, occurring in clusters of bursts separated by relatively long closed periods (Inagaki et al., 1995). Time constants for the open times and closed times within the bursts are ~ 2 to 3 ms, and 0.3 to 0.5 ms, respectively.

1.2.2. Regulation of K_{ATP} channels by ATP and ADP

When applied to the cytosolic side of the membrane, ATP inhibits K_{ATP} channels in a dose-dependent manner. A complete block of K_{ATP} channels is observed at millimolar concentrations of ATP, which affects channel kinetics by reducing the number of openings per burst and increasing the duration of the closed intervals between bursts. ATP has no effect on the amplitude of single-channel currents (Nelson and Quayle, 1995).

ATP has two effects on K_{ATP} channels: inhibiting channels activity and maintaining the channel in an operative state (Cao et al., 2002). Since the inhibitory effect can be observed also with nonhydrolyzable analogues of ATP such as adenylyl imidodiphosphate (AMP-PNP) and adenylylmethylenediphosphate (AMP-PCP), and by ATP in the absence of Mg^{2+} (ATP^{4-}), it has been thought that the hydrolysis of ATP and phosphorylation of the K_{ATP} channel is not necessary for the inhibition by ATP. One important property of K_{ATP} channels is the gradual inactivation that follows patch excision in an ATP-free medium; a process known as “channel run down” (Okuyama et al., 1998). Exposure of the patch to MgATP can prevent a rundown of the K_{ATP} channels and also can “refresh the activity” of the rundown K_{ATP} channels. Furthermore, nonhydrolyzable ATP analogues do not substitute for MgATP in the maintenance of K_{ATP} channel activity. These findings suggest that hydrolysis of ATP or phosphorylation of the K_{ATP} channel is required to maintain the channels in an operative state.

On the other hand, the inhibitory action of ATP is reduced in the presence of MgADP. The cytoplasmic ATP/ADP ratio that reflects the metabolic status of the cell is thought to be more important for the regulation of K_{ATP} channels than the ATP concentration by itself in regulating channel activity in intact cells. The stimulatory and

inhibitory effects of the nucleotides on the K_{ATP} channels have been proposed to be mediated through two nucleotide-binding sites present in K_{ATP} channel. A high concentration of ADP in the absence of Mg^{2+} (ADP^{3-}), when applied to the cytoplasmic surface of excised membrane patches can inhibit K_{ATP} channel activity, suggesting that ADP^{3-} binds to the same site as ATP^{4-} does in the K_{ATP} channel.

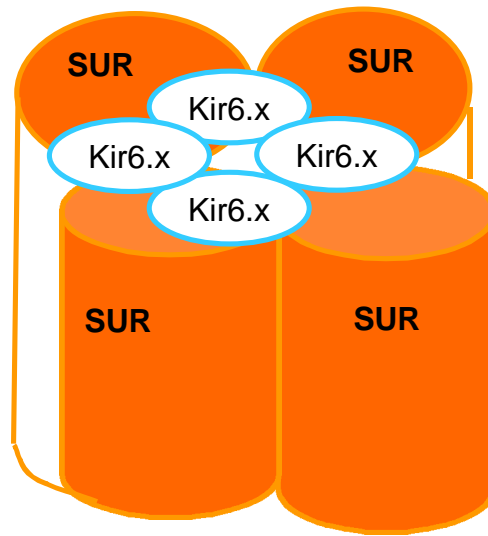
1.3. MOLECULAR STRUCTURE AND FUNCTIONAL DIVERSITY OF K_{ATP} CHANNELS

1.3.1. The inwardly rectifying K^+ channel family

Inwardly rectifying K^+ channels are distinct from voltage-gated K^+ (K_v) channels because they are not activated by membrane depolarization and because they allow a larger K^+ influx than they do an efflux (Jan and Jan, 1997). However, the structure of the inwardly rectifying K^+ channel was unknown until 1993.

In 1993, Ho et al. and Kubo et al. reported the expression cloning of cDNA encoding distinct inwardly rectifying K^+ channels. These studies led to the elucidation of the structure and function of the subfamily of inwardly rectifying K^+ channels. The inwardly rectifying K^+ channel proteins so far identified vary from ~ 360 to 500 amino acids long, and the amino acid identity is ~ 40% between members belonging to different subfamilies and ~ 60% between individual members within each subfamily. At least 7 subfamilies are now identified designated Kir1.0 - 7.0, in the inwardly rectifying K^+ channel family (Okuyama et al., 1998). A hydropathy profile of the proteins predicts the presence of two transmembrane domains (M1 and M2) in Kir channels (Fig. 1.2), a feature different from K_v channels, which have six transmembrane domains (Ho et al.,

A.



B.

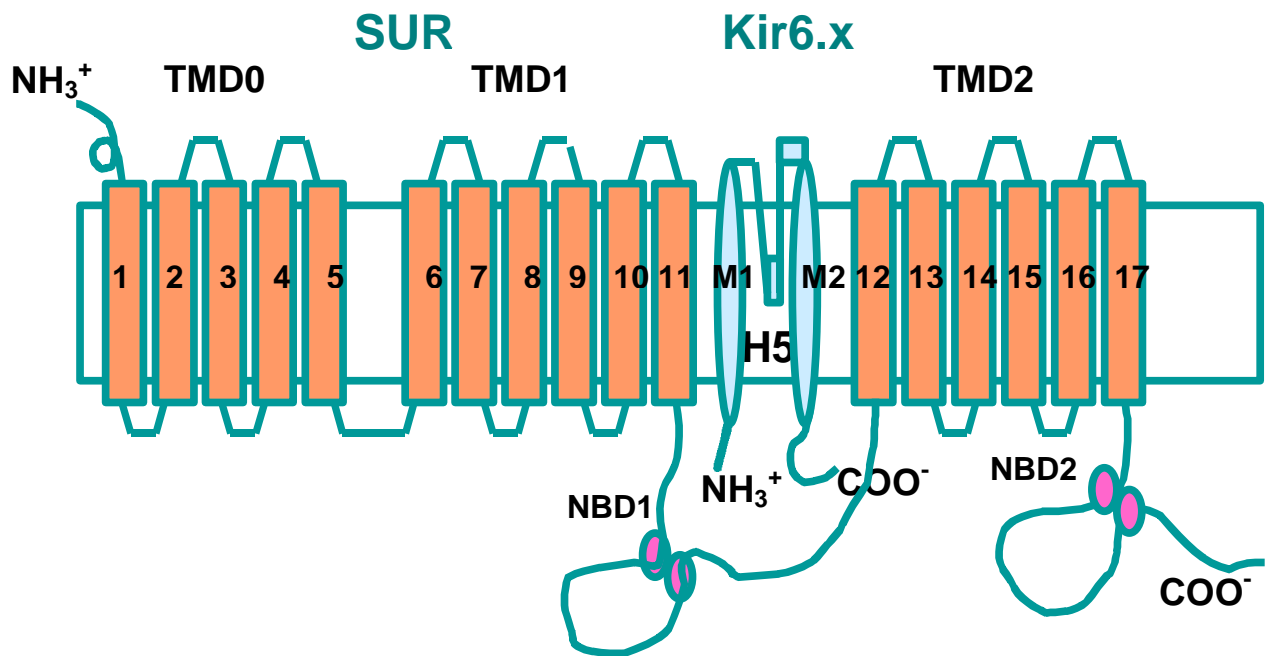


Figure 1.2. Molecular structure of K_{ATP} channel. (A) Assembly of K_{ATP} channel. The K_{ATP} channel comprises of two subunits: Kir6.x (Kir6.1 or Kir6.2) and the regulatory subunit sulphonylurea receptor SUR (SUR1 or SUR2). (B) Membrane topology of SUR and Kir6.x. The sulphonylurea receptor has been proposed to have seventeen transmembrane domains. Kir6.x has two transmembrane domains (M1 and M2). Abbreviations: NBD1 and NBD2, nucleotide binding domains 1 and 2; TMD, transmembrane domains). (Gros et al., 2002)

1993). However, Kir6.x channels retain the highly conserved H5 (or P) region between the two transmembrane domains (Yang et al., 1995; Ashcroft and Gribble, 1998).

Hydropathy analysis and epitope labelling suggests that SUR subunits have seventeen transmembrane domains (TMDs). These may be grouped into three sets (TMD 0, 1, 2), consisting of 5, 6 and 6 transmembrane domains, respectively (Cao et al., 2002). Like other ABC transporters, SUR has two large cytosolic loops referred to as nucleotide binding domains (NBD1 and NBD2) that contain consensus sequences for Mg-nucleotide binding and hydrolysis. Interaction of nucleotides with the NBDs stimulates channel activity. The sulphonylurea receptor SUR contains the binding sites for sulphonylureas and KCOs and endows the K_{ATP} channel with sensitivity to both groups of drugs. Kir6.x has no obvious consensus site for nucleotide binding; nevertheless, it is now clear that Kir6.x forms the K_{ATP} channel pore and contains the site at which ATP mediates channel inhibition. Considering its high degree of amino acid sequence similarity to the H5 region of Kv channels, it is likely that the H5 region of Kir channels also is responsible for K^+ selectivity. Several lines of evidence indicate that the inwardly rectifying K^+ channels are composed of homomeric or heteromeric tetramers of the channel subunits (Sun et al., 2004).

Table 1.1. Reconstitution of K_{ATP} channels from SUR subunit and Kir6.x subunit

Type of K _{ATP} channel	Subunit combination	Properties		
		ATP sensitivity	Glibenclamide sensitivity	Diazoxide sensitivity
Vascular smooth muscle type ¹	SUR2B+Kir6.1	(-)	(+)	undetermined
Retinal glial cells ²	SUR1+Kir6.1	(+)	undetermined	undetermined
	SUR2A+Kir6.1	(+)	undetermined	undetermined
Smooth muscle type ³	SUR2B+Kir6.2	(+) low	(+)	(+)
Cardiac and skeletal muscle type ⁴	SUR2A+Kir6.2	(+) low	(+) low	(-)
Pancreatic β -cell type ⁵	SUR1+Kir6.2	(+) high	(+) high	(+)

References:

¹ Yamada et al., 1997² Skatchkov et al., 2001³ Isomoto et al., 1996⁴ Inagaki et al., 1996⁵ Inagaki et al., 1995; Sakura et al., 1995

1.3.2. Kir6.1 channels

Inwardly rectifying K^+ channels allow K^+ ions to enter the cell much more readily than they do K^+ permeation in the opposite direction, regardless of the K^+ concentration in the external solution and hence of the K^+ equilibrium potential. Thus, inwardly rectifying potassium channels appear to be sensitive to the electrophysiological driving force for K^+ ions. These channels play a significant role in determining the resting potential of the cell and may be sensitive to the electrochemical driving force for K^+ ions (Ammala et al., 1996a).

Inwardly rectifying K^+ channels (Kir) have many important physiological roles. Based on sequence similarity, the Kir family currently is divided into seven subfamilies, Kir1.x through Kir7.x. Kir6.x subfamily members, including Kir6.1 (originally termed uK_{ATP}) encoded by potassium inwardly-rectifying channel, subfamily J, member 8 (*Kcnj8*) and Kir6.2 (originally termed BIR) encoded by potassium inwardly-rectifying channel, subfamily J, member 11 (*Kcnj11*). There are two isoforms of the gene encoding SUR, SUR1 (also known as Abcc8) and SUR2 (Abcc9). In addition, there are several splice variants of SUR2, the major ones being SUR2A and SUR2B. Reconstitution studies have shown that different combinations of Kir6.1 or Kir6.2 and SUR1 or SUR2 variants generate K^+ currents (Table 1.1) with distinct nucleotide sensitivity and pharmacological properties (Giblin et al., 2002a,b).

Although the electrophysiological and pharmacological properties of Kir6.2-containing K^+ channels have been well characterized in reconstituted systems and native tissues, those of Kir6.1-containing K^+ channels are not fully understood. Co-expression of Kir6.1 and SUR1 in HEK-293 cells produces tolbutamide-sensitive K^+ channel currents, whereas co-expression of Kir6.1 and SUR2A in COS7 cells produces currents

responsive to very high concentrations of nucleoside diphosphate (UDP); however, their physiological significance is unknown (Giblin et al., 2002a,b). Co-expression of Kir6.1 and SUR2B in HEK-293T cells produces a K^+ current activated by nucleotide diphosphates such as UDP and inhibited by the sulphonylurea glibenclamide. These properties are similar to those of the K_{ATP} channels in native vascular smooth muscle (vascular K_{ATP} channel) (Ammala et al., 1996a). Because K^+ channel openers (KCOs) with vasodilating effects, such as cromakalim and pinacidil, open the vascular K_{ATP} channels, the channels are thought to be involved in the vasodilation response of vascular smooth muscle. To clarify the physiological roles of Kir6.1-containing channels directly, mice lacking the gene encoding Kir6.1 (*Kcnj8*) were generated. It was shown that the Kir6.1-containing channel is critical in regulation of vascular tones, and that its genetic disruption in mice causes sudden cardiac death (SCD) associated with arrhythmia and atrioventricular (AV) block caused by spontaneous cardiac ischemia, a phenotype resembling that of Prinzmetal (or variant) angina in humans (Seino and Miki, 2003). SCD is thought to be the principle cause of unexpected, rapid death in adults and infants. Although most SCD occurs in patients with cardiac disease such as coronary atherosclerosis, myocardial disease and inflammatory cardiac disease, some occurs unexpectedly from abrupt cardiac arrest due to arrhythmia or myocardial ischemia. Genetic abnormalities of ion channels (K^+ , Na^+ or Ca^{2+} channel subunits), a gap junction protein (connexin40) and cardiac-specific transcription factors have so far been shown to be involved in SCD caused by lethal arrhythmia in human and mice. Of these, abnormalities in K^+ , Na^+ and Ca^{2+} channel affect depolarization and repolarization of cardiomyocytes and cause long-QT syndrome in human and mice. In cardiomyocytes isolated from mouse models of long-QT syndrome, abnormal ion currents were detected

on the plasma membrane. In Kir6.1-null mice, however, no electrophysiological abnormalities were found in cardiomyocytes. In addition, there were normal plasma membrane K_{ATP} channels in the cardiomyocytes of Kir6.1-null mice. Accordingly, Kir6.1 is unlikely a constituent of the plasma membrane K_{ATP} channels in cardiomyocytes. Together with the previous finding of the absence of plasma membrane K_{ATP} channels in the cardiomyocytes of Kir6.2-deficient (Kir6.2-null) mice, this suggests that Kir6.1-Kir6.2 heteromultimers do not form *in vivo*, or are of no functional significance, as suggested by *in vitro* experiments (Seino and Miki, 2003). The lack of abnormalities in the electrophysiological properties of cardiomyocytes of Kir6.1-null mice indicates that the pathophysiology of the atrioventricular (AV) block in Kir6.1-null mice differs from that in long-QT syndrome due to K^+ , Na^+ or Ca^{2+} channel dysfunction. Alternatively, the AV block is caused by the spontaneous ST elevation due to the myocardial ischemia. In contrast to the normal electrophysiological properties of Kir6.1-null myocytes, it has been found that in vascular smooth muscle K_{ATP} channels are defective in aortas of Kir6.1-null mice. As the K_{ATP} channel in smooth muscle of the aorta is intact in Kir6.2-deficient mice, these findings indicate that Kir6.1 is a constituent of the K_{ATP} channels of the plasma membrane in vascular smooth muscle. The lack of vasodilation response to pinacidil both *in vivo* (blood pressure decrease) and *in vitro* (relaxation of the aortic rings) indicates that Kir6.1-containing K_{ATP} channels have a critical role in the regulation of vascular tonus.

The most remarkable finding in Kir6.1-null mice is spontaneous coronary spasm leading to lethal AV block, a phenotype resembling that of Prinzmetal (or variant angina) in humans. Prinzmetal angina is an unusual form of unstable angina reported by Prinzmetal et al. in 1959, which occurs almost exclusively at rest and is associated with

elevation of ST segments on electrocardiogram (ECG) during the attack. The pathophysiology of Prinzmetal angina is thought to include hypercontractility of epicardial coronary arteries with or without atherosclerotic changes. Although the attack disappears spontaneously in most cases, it can lead to myocardial infarction, AV block, life-threatening ventricular tachycardia and sudden death if the coronary vasospasm is prolonged. Prinzmetal angina is diagnosed by elevated ST segments on ECG during the attack or by induction of coronary spasm using ergot alkaloids or acetylcholine. Because the sudden death of Kir6.1-null mice is associated with spontaneous ST elevations and/or AV block, and administration of the ergot alkaloid methylergometrine elicited significant elevation of ST segments both *in vivo* and *in vitro*, Kir6.1-null mice can be proposed as an animal model of Prinzmetal angina. The depressed ST segments seen in Kir6.1-null mice likely reflect coronary artery spasm at different sites. The findings that coronary vascular resistance is elevated under basal conditions and increased markedly after methylergometrine application in Langendorff perfused heart preparation from Kir6.1-null mice demonstrate directly that the disruption of Kir6.1 causes the coronary spasm responsible for the phenotype of Kir6.1-null mice.

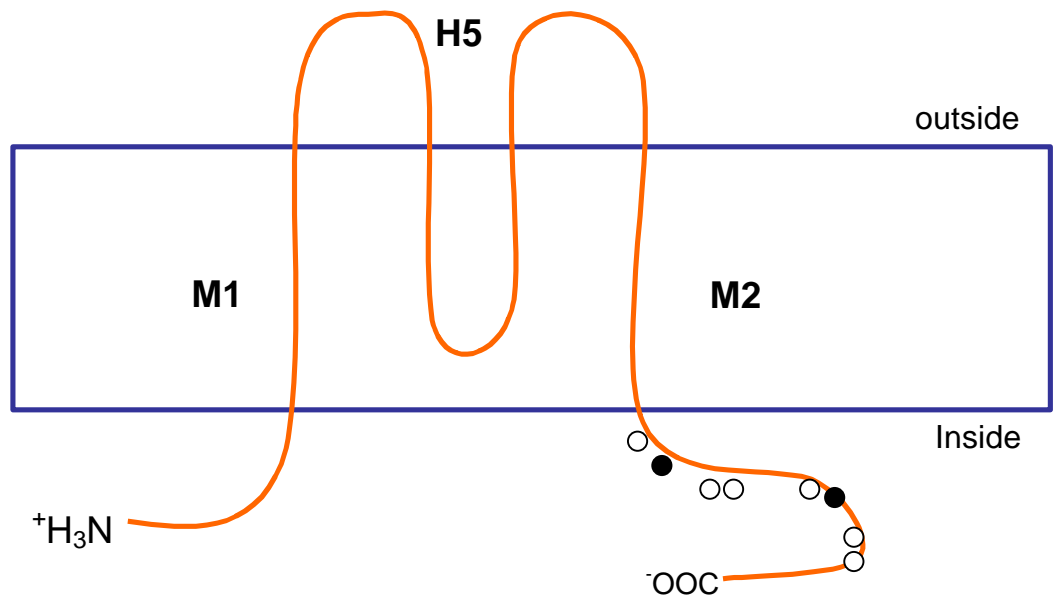
1.3.3. Kir6.1 channel structure

Using GIRK1 (Kir3.1) cDNA as a probe, researchers cloned uK_{ATP}-1 (currently referred to as Kir6.1) from a rat pancreatic islet cDNA library. Rat Kir6.1 is a 424-amino acid protein that has two putative transmembrane segments (Aguilar-Bryan et al., 1998) (Fig. 1.3). As Kir6.1 shares only 40-50 % identity with previously cloned Kir channel members, it represents a new subfamily, Kir6.x. The glycine-tyrosine-glycine motif in the H5 region, which is critical for K⁺ ion selectivity and is highly conserved among K⁺

channels, is not conserved in Kir6.1. The motif in Kir6.1 is glycine-phenylalanine-glycine (G-F-G). The intracellular C-terminal region in human Kir6.1 has two potential protein kinase A-dependent phosphorylation sites (threonine-234 and serine-385) and seven protein kinase C-dependent phosphorylation sites (serine-224, threonine-345, serine-379, serine-385, serine-391, and serine-397). Kir6.1 is expressed ubiquitously, but it is not expressed in the insulin-secreting cell line HIT-T15 (hamster derived), RINm5F (rat-derived), and MIN6 (mouse-derived), all of which have K_{ATP} channels. Studies using immunoblot analysis of subcellular fractions and electron microscope examination showed that Kir6.1 is present predominantly in the inner membrane of mitochondria (Seharaseyon et al., 2000; Hu et al., 1999) of cardiac myocytes. Kir6.1 protein in cardiac myocytes is upregulated by pinacidil, and expression of the Kir6.1 gene is induced by cardiac ischemia (Akao et al., 1997). The human Kir6.1 gene (*Kcnj8*) is mapped to chromosome 12p11.23.

An isoform of Kir6.1, BIR (the β -cell inward rectifier, currently referred to as Kir6.2), was subsequently cloned from a human genomic library and the MIN6 cDNA library, using Kir6.1 cDNA as a probe. Kir6.2 is a 390-amino acid protein that shares 71% amino acid identity with Kir6.1. Like Kir6.1, Kir6.2 has the glycine-phenylalanine-glycine motif in the H5 region. Both Kir6.1 (residue 170) and Kir6.2 (residue 160) have asparagine at the corresponding position, as is found in the weak Kir channel subunit Kir1.1 (residue 171).

A. Kir6.1



B. Kir6.2

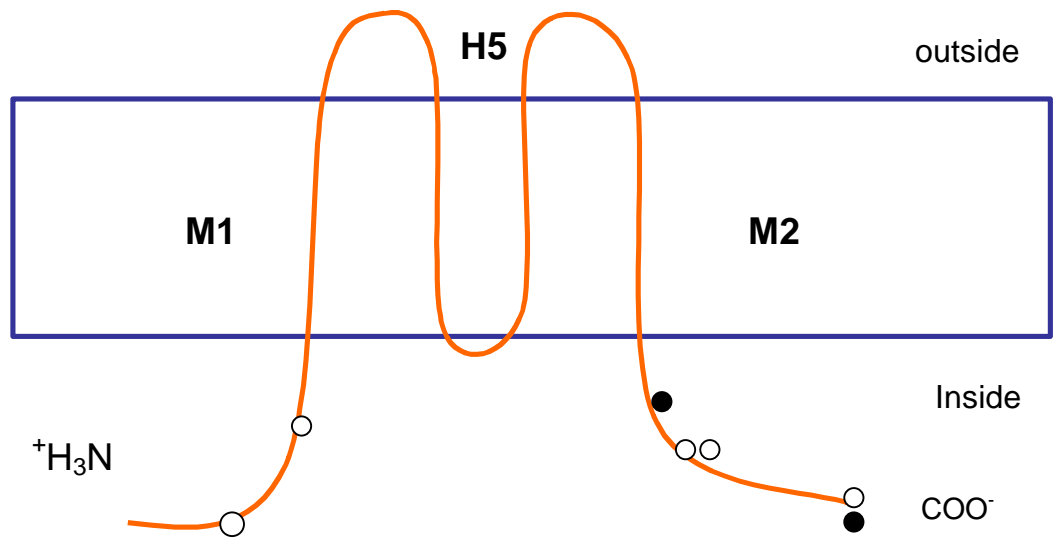


Figure 1.3. Proposed membrane topology of Kir6.1 (A) and Kir6.2 (B). Putative transmembrane segments (M1, M2) and pore forming region (H5) are shown. Potential protein kinase A- and protein kinase C-dependent phosphorylation sites (in human Kir6.1 and Kir6.2) are indicated by filled circles and open circles, respectively. NH^{3+} and COO^- indicate the N- and C terminus, respectively.

1.3.4. Reconstitution of K_{ATP} channels

Reconstitution studies with a Kir6.x subfamily subunit and a SUR subunit in heterologous expression systems have suggested that different combinations of the Kir6.1 or Kir6.2 subunits and the SUR1, SUR2, or their variant subunits (Brochiero et al., 2002; Noulin et al., 2001) could account in part for the molecular and functional diversity of K_{ATP} channels (Table 1.1).

1.3.5. Reconstitution from the Kir6.1 and SUR1 subunits

Early reports indicated that Kir6.1 alone, when expressed heterologously in HEK-293 cells, elicited ATP-sensitive K⁺ channel currents, although the currents were not sensitive to sulphonylureas and diazoxide (Ammala et al., 1996b). However, recent studies show that SUR1 is required for ATP, sulphonylurea, and diazoxide sensitivities and for functional expression of Kir6.1 currents when *Xenopus oocytes* (Ammala et al., 1996a) or COSm6 cells (Clement et al., 1997) are used as expression systems. These observations suggest that the activity of the Kir6.1 channel requires a sulphonylurea receptor or other modulator of the Kir6.1 channel that is present endogenously in HEK-293 cells and not in *Xenopus oocytes* or COSm6 cells (Ammala et al., 1996a). Another possibility is that Kir6.1 requires another subunit of the Kir channel family that is expressed endogenously in HEK-293 cells to form a functional K⁺ channel as a heteromultimer. Kir6.1 subunit plus the SUR1 subunit can reconstitute ATP-, tolbutamide-, and diazoxide-sensitive K⁺ channel currents (Ammala et al., 1996a). It has previously been shown with immunocytochemistry studies that retinal glial cells in adult frogs express Kir6.1 and SUR1, while retinal neurons display Kir6.2 and SUR2A/B (Skatchkov et al., 2002). Using immunocytochemistry and electrophysiology, the

expression of Kir6.1/SUR1 (K_{ATP}) channels in adult frog and tadpole Muller cells has been demonstrated. Using conditions favoring the activation of K_{ATP} channels (i.e., ATP- and spermine-free cytoplasm-dialyzing solution containing gluconate) in Muller cells isolated from both adult frogs and tadpoles, Skatchkov et al. (2002) demonstrated the following. First, using the whole-cell configuration of patch-clamp technique, they showed that tolbutamide, a blocker of K_{ATP} channels, blocks nearly 100% of the transient and about 30% of the steady-state inward currents and depolarizes the cell membrane by 5-12 mV. Second, inside-out membrane patches display a single-channel inward current induced by gluconate (40 mM) and blocked by ATP (200 μ M) at the cytoplasmic side. The channels apparently show two sublevels (each of approximately 27-32 pS) with a total of 85 pS maximal conductance at -80 mV. Thus, functional K_{ATP} channels, composed of Kir6.1/SUR1, are present in frog Muller cells and contribute a significant part to the whole-cell K^+ inward currents in the absence of ATP. Other inwardly rectifying channels, such as Kir4.1 or Kir2.1, may mediate the remaining currents. K_{ATP} channels may help maintain glial cell functions during ATP deficiency (Skatchkov et al., 2002). Skatchkov et al. (2001) had shown using immunocytochemistry in frog retina that most members of the Kir subfamily are expressed in specific neuronal compartments. However, Kir 6.1, the pore-forming subunit of K_{ATP} channels, is expressed exclusively in glial Muller cells. Muller cell endfeet display strong Kir 6.1 immunolabel throughout the retina, whereas the somata are labeled only in the retinal periphery. The co-expression of Kir 4.1 and Kir 6.1 subunits may enable the cells to maintain their high K^+ conductance and hyperpolarized membrane potentials both at high ATP levels (Kir 4.1) and during ATP deficiency (Kir 6.1) (Skatchkov et al., 2001).

1.3.6. Reconstitution from the Kir6.1 and SUR2B subunits

Coexpression of Kir6.1 and SUR2B produces K⁺ channel currents with unitary conductance of approximately 33 pS in the presence of pinacidil. The activity of SUR2B/Kir6.1 channel is inhibited by ATP only at high concentrations ($> 10^{-4}$ M) but is stimulated at lower concentrations (10^{-6} to $\sim 10^{-4}$ M). Similarly, GTP stimulates and inhibits channel activity at higher concentrations ($>10^{-4}$ M) and lower concentrations (10^{-6} to $\sim 10^{-4}$ M), respectively. SUR2B/Kir6.1 channel activity is stimulated by the K⁺ channel openers pinacidil and nicorandil. Glibenclamide inhibits nicorandil-activated K⁺ channel currents. Nucleoside diphosphates such as UDP and GDP also stimulate SUR2B/Kir6.1 channel activity (Nelson and Quayle, 1995).

The Kir6.1/SUR2B channel closely resembles a K⁺ channel found in vascular smooth muscle cells which is sensitive to KCO drugs but not to ATP (Kajioka et al., 1991; Nelson and Quayle, 1995). This channel has a conductance similar to that of the Kir6.1/SUR2B channel (30-50 pS in symmetrical ~ 145 mM K⁺ solutions), is spontaneously closed in excised membrane patches, is activated by internal nucleotide di- and triphosphate, and shows little or no inhibition with internal ATP. In order to distinguish it from the classical K_{ATP} channels, this channel is described as the nucleotide diphosphate-dependent K⁺ (K_{NDP}) channel (Beech et al., 1993). Because the major effect of KCO drugs *in vivo* is vasodilatation, many drugs with these profiles have been synthesized as novel antihypertensive and/or coronary vasodilators (Weston and Edwards, 1992). Since cardiac and pancreatic K_{ATP} channels were the first to be identified as targets for KCO drugs, the fact that the K_{ATP} channel lacks ATP sensitivity has posed a dilemma for the identification of the KCO drug target in smooth muscle cells (Weston and Edwards, 1992). This discrepancy may have occurred because the

K_{ATP} channel and K_{NDP} channels are structurally related. In particular they share the SUR, which appears to be responsible for the common pharmacology of the channels, while differing in their Kir subtypes, which provokes an entirely different sort of response to intracellular nucleoside triphosphate as well as single-channel behavior.

1.4. NICOTINE

1.4.1. Introduction to nicotine

Chemicals with behavioral and physiological activity are delivered to tobacco users when they smoke a cigarette or use other tobacco products. Whether these chemicals are absorbed in quantities that are of biological significance and whether such absorption is related to the behavior of the tobacco users are critical issues in understanding their role in addictive tobacco use. The scientific study of the absorption process, distribution within the body, and elimination from the body of drugs and chemicals is called pharmacokinetics. The study of drug and other chemical actions on the body, over time, is called pharmacodynamics. The pharmacokinetics and pharmacodynamics of some tobacco smoke constituents, particularly nicotine, have been extensively studied. These studies show an orderly relationship between the use of tobacco and the absorption of nicotine. Research shows that nicotine is well absorbed from tobacco; that it is distributed rapidly and in biologically active concentrations to body organs, including the brain; and that nicotine is the major cause of the predominant behavioral effects of tobacco and some of its physiologic consequences. One effect of nicotine, development of tolerance to its own actions, is similar to that produced by other addictive drugs. Tolerance refers to decreasing responsiveness to a drug or

chemical such that larger doses are required to produce the same magnitude of effect. Tolerance to many actions of nicotine occurs in animals and humans.

Smoking adversely affects the cardiovascular system in human subjects. Smoking is associated with an increased risk of atherosclerotic vascular disease, hypertension, myocardial infarction, unstable angina, sudden cardiac death, and stroke (Winniford, 1990). The adverse effects of smoking on vascular function have been examined in human subjects. These studies have shown that acute and chronic cigarette smoking impairs nitric oxide synthase-mediated relaxation of large blood vessels (Zeiber et al., 1995). In addition, it appears that cessation of smoking is associated with improvement of endothelial function in human subjects. Mechanisms that contribute to impaired endothelium-dependent relaxation of large blood vessels during cigarette smoking have been investigated. Studies by Motoyama et al. (1997) and Heitzer et al. (1996) found that impaired endothelium-dependent vasodilatation observed in chronic smokers could be restored by acute treatment with vitamin C, an antioxidant. Thus it appears that oxygen radical formation plays an important role in impaired reactivity of large conduit vessels in chronic smokers (Ting et al., 1997).

Studies using animal models have shown that smoking and the components of cigarette smoke impair endothelium-dependent responses of blood vessels (Suzuki et al., 1996a; Ota et al., 1997). Mechanisms that contribute to impaired endothelium-dependent reactivity of blood vessels after exposure to cigarette smoke also have been examined. It has been found that cigarette smoke extract impairment of endothelium-dependent dilatation of cheek pouch arterioles could be restored by treatment with indomethacin, a cyclooxygenase inhibitor. These findings suggest that the mechanism of impaired responses of cheek pouch arterioles after exposure to cigarette smoke extract appears to

be related to the release of substances produced via the cyclooxygenase pathway (Rubinstein et al., 1991). This was confirmed by Suzuki et al. (1996a) who found that smokeless tobacco (moist snuff) impaired endothelium-dependent dilatation of cheek pouch arterioles. Indomethacin and SQ-29548, a thromboxane A₂/prostaglandin H₂-receptor antagonist, abrogated the attenuating effects of smokeless tobacco extract on acetylcholine- and bradykinin-induced vasodilation. These data indicate that an aqueous extract of smokeless tobacco impairs endothelium-dependent vasodilation in the oral mucosa *in situ* in a specific fashion and that these effects are mediated, in part, by cyclooxygenase products of arachidonic acid metabolism that stimulate thromboxane A₂/prostaglandin H₂ receptors. Cigarette smoking is one of the risk factors for hypertension and stroke. However, a consensus of the causal relationship between cardiovascular disorders and consumption of smokeless tobacco has not been reached yet. What has been known is the acute hypertensive effect of smokeless tobacco on users (Westman 1995; Nowak, 1994). During smokeless tobacco use (5 to 30 min), systolic and diastolic blood pressure persistently increased. This increase remained up to 90 min after smokeless tobacco use. The potential long-term risk of using smokeless tobacco is obvious since smokeless tobacco induced transient hypertension may predispose smokeless tobacco users to myocardial infarction, hypertension, and stroke (Benowitz, 1993). The most significant substance that raises blood pressure in smokeless tobacco is nicotine. Yet the vascular effects of nicotine and the underlying mechanisms are still to be clarified. Nicotine, in smokeless tobacco, is absorbed rapidly through the oral mucosa in smokeless tobacco users. Absorbed nicotine releases catecholamine from sympathetic nerve endings. The subsequent activation of α -

adrenoceptors in vascular smooth muscle cells contracts vascular tissues and elevates blood pressure (Toda et al., 1995). Nicotine also acts directly on vascular smooth muscle cells to induce vascular relaxation or contraction. The direct effect of nicotine on vascular smooth muscle cells as well as the nicotine-induced adrenergic stimulation substantially contributes to altered cardiovascular function with smokeless tobacco consumption.

1.4.2. Nicotine and other alkaloids in various tobacco products

Nicotine is a tertiary amine composed of a pyridine and a pyrrolidine ring (Fig. 1.4). Nicotine may exist in two different three dimensionally structured shapes, called stereoisomers. Tobacco contains only (S)-nicotine (also called l-nicotine), which is the most pharmacologically active form. Tobacco smoke also contains the less potent (R)-nicotine (also called d-nicotine) in quantities up to 10 percent of the total nicotine present. Although the major alkaloid in tobacco is nicotine, there are other alkaloids in tobacco. These include nornicotine, anabasine, myosmine, nicotyrine, and anatabine. These substances make up 8 to 12 percent of the total alkaloid content of tobacco products.

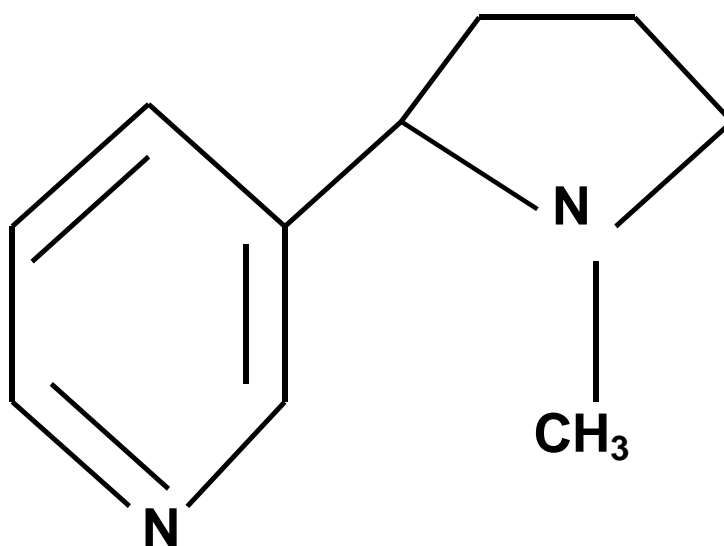


Figure 1.4. Structure of nicotine. Nicotine is a tertiary amine composed of a pyridine and a pyrrolidine ring.

1.4.3. Effect of nicotine on resistant arteriolar dilatation

In vivo, acute infusion of nicotine at low concentrations causes impairment of the endothelium-dependent dilatation of peripheral resistance arterioles contained within the microcirculation of the hamster cheek pouch (Mayhan and Patel 1997). High concentrations of plasma nicotine (14 ng/ml), which is similar to that observed in smokers, produced a profound selective impairment of endothelium-dependent vasodilatation.

The effect of nicotine on endothelium-dependent vasodilatation is reversed by topical application of superoxide dismutase (SOD). Therefore, it has been suggested that nicotine impairs endothelium-dependent arteriolar dilatation via an increase in the synthesis/release of oxygen-derived free radicals (OFR) (Mayhan and Sharpe 1998). Cigarette smoke and products of cigarette smoke produce diffuse vascular injury in many organ systems and impair nitric oxide synthase-dependent dilatation of large peripheral arteries (Celermajer et al., 1993) and resistance arterioles (Rubinstein et al., 1991). Investigators have shown that nicotine has toxic effects on endothelium, and thus nicotine may play a key role in impaired nitric oxide synthase-dependent vasoreactivity observed in users of tobacco. Nicotine produces morphological abnormalities of the endothelium causing direct alteration of the vascular reactivity. Heitzer et al. (1996) reported that treatment of smokers with vitamin C, an antioxidant, improved impaired endothelium-dependent reactivity of large and peripheral arteries (Fig. 1.5). In animals planted with nicotine containing pumps, it has been shown that plasma levels were strongly related to the dose of nicotine administered (0.18-4.7 mg/kg/day). There was a strong positive correlation between plasma levels of nicotine and its major metabolite cotinine in nicotine treated rats. It is probable that nicotine concentration in

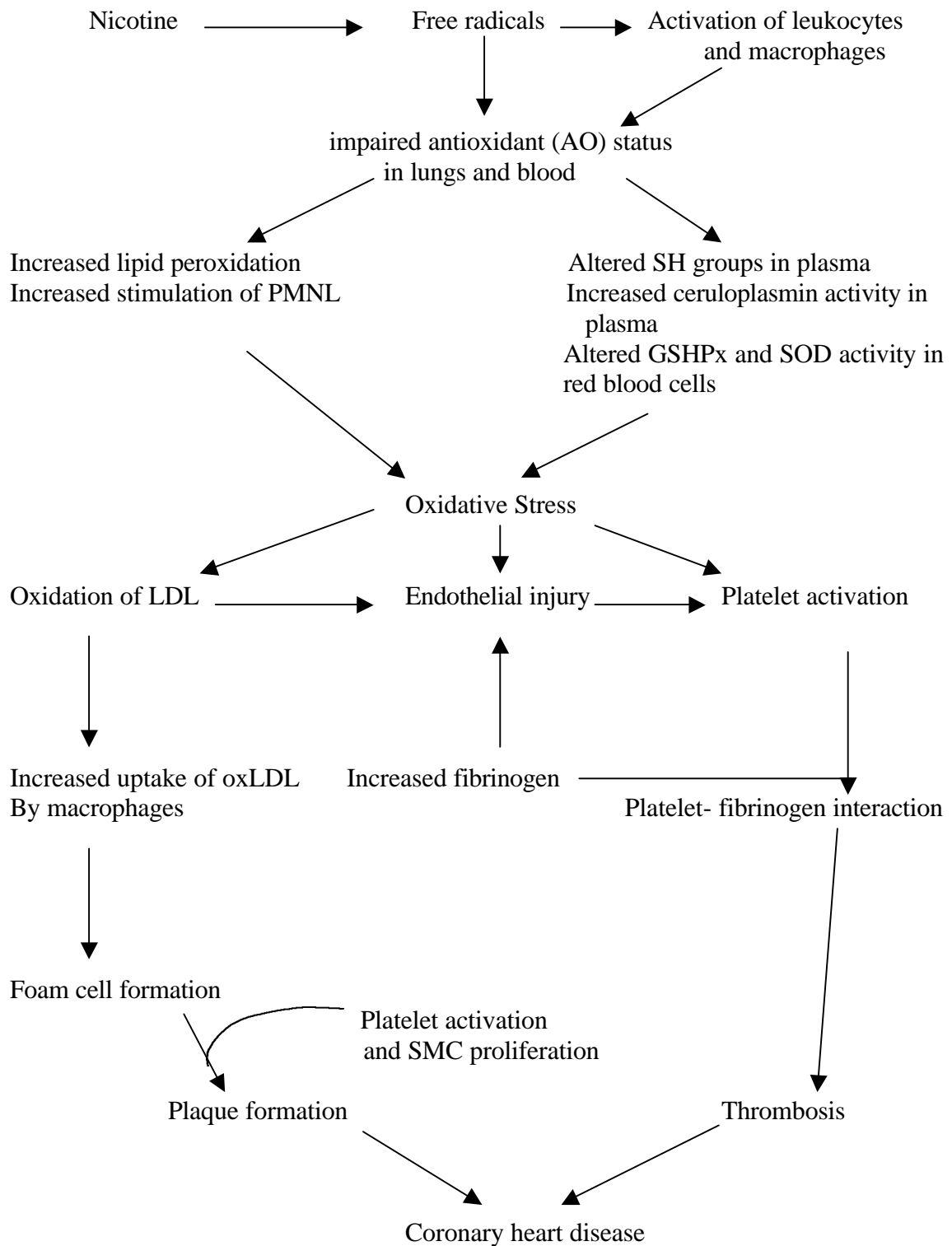


Figure 1.5. Schematic diagram of oxidative stress in smoking leading to heart disease (Olinescu and Smith 2002).

habitual smokers is elevated throughout the course of the day. In support of this, Isaac and Rand (1972) have shown that the plasma level of nicotine is 25 ± 6 ng/ml at 6.5 h after *ad libitum* smoking. Furthermore, several previous studies have shown that plasma levels of nicotine are elevated in chronic smokers (Benowitz, 1993, Hirshkowitz et al., 1992; Motoyama et al., 1997). Thus, although many of the above studies examined the acute effects of nicotine infusion on arteriolar reactivity, it is believed that those studies have important implications for the chronic effects of cigarette smoking and the use of smokeless tobacco products on vascular reactivity because this might more accurately reflect what is found in chronic smokers. And indeed, it has been shown that in hamsters chronically treated with nicotine (2 μ g/kg/day) for 2-3 weeks, the resistance arterioles of the cheek pouch produces a selective impairment of endothelium-dependent arteriolar dilatation via a mechanism related to the synthesis/release of OFR (Mayhan and Sharpe, 1999). The magnitude of the resistant arteriole vasodilatation in response to acetylcholine was significantly less in hamsters treated with nicotine than in control hamsters. Superfusion with SOD potentiates responses of the arterioles to acetylcholine. Thus it appears that SOD, although it does not affect the baseline diameter of resistant arterioles, can prevent the impairment of endothelium-dependent arteriolar reactivity in hamsters treated with nicotine. Although these previous studies provide insights into the mechanisms by which acute and chronic treatment of nicotine could alter the endothelium-dependent reactivity of resistance vessels, there are other studies that showed nicotine might not have altered acetylcholine-induced changes in perfusion pressure of isolated mesenteric circulation. Li and colleagues (1994) examined the pressure drop across the isolated perfused mesenteric arteries in rats in response to

acetylcholine. These investigators found that chronic (2-weeks) treatment with nicotine did not alter acetylcholine-induced changes in perfusion pressure of isolated rat mesenteric circulation.

1.4.4. Nicotine and ion channels

Nicotine is the main constituent of tobacco smoke responsible for the elevated risk of the cardiovascular disease and sudden coronary death associated with smoking, presumably by provoking cardiac arrhythmias. The cellular mechanisms may be related to the ability of nicotine to prolong action potentials and depolarize membrane potential. However, the underlying ionic mechanisms remained unknown. Wang and colleagues (2000a) demonstrated that nicotine blocked multiple types of K^+ currents (including A-type currents, delayed rectifier current and inward rectifier current) with preferential inhibition of transient outward currents $I_{to}/Kv4.3$. Those results indicate that nicotine is a non-specific blocker of K^+ channels with certain selectivity toward A-type currents. The effects of nicotine were independent of nicotine receptor stimulation or catecholamine release since the effects were not reversed or prevented by mecamylamine (100 μ M, nicotine receptor antagonist), atropine (1 μ M, muscarinic receptor antagonist), or propranolol (2 μ M, non-selective β -adrenoceptor blocker). Thus the inhibitory effects are likely the consequence of direct interactions between nicotine molecules and the channel protein. Reports on the effect of nicotine on ion channels are not conclusive. Wang et al. (2000a) provided insight into potential mechanisms underlying nicotine block of A-type currents. Approximately 40% of the total inhibition could be ascribed to tonic block, with the remainder (60%) due to use-dependent block, for both $Kv4.3$ and

I_{to} . This would imply that nicotine binds to transient outward K^+ current (I_{to}) channels in the closed state.

The cardiac effects of nicotine have been ascribed to enhanced release of catecholamines. However, accumulating evidence has shown that nicotine can also exert its effects without involvement of nAChRs and catecholamine release. Studies under conditions devoid of nAChR stimulation demonstrated the ability of nicotine to alter action potential (AP) characteristics in guinea pigs (Pappano and Rembush 1970), rabbits (Carryl et al., 1992), and dogs (Greenspan et al., 1969) in different tissues such as sinus nodes (Satoh, 1997), atrium, ventricle, and Purkinje fibers. The most noticeable changes were decreases in resting potential and prolongation of later AP phases. It is therefore quite conceivable that nicotine might be able to interact directly with ion channels. Hamon et al. (1997) first reported that nicotine inhibited slowly inactivating K^+ currents in rat cultured striatal neurons. The effects were attributed to stimulation of nicotine receptors, because the nicotinic antagonist dihydro- β -erythroidine reversed and nicotinic agonists reproduced the block. Direct effects on K^+ channels were not revealed until recently by our laboratory (Tang et al., 1999) in experiments that used vascular smooth muscle cells. Tang et al. (1999) demonstrated that nicotine caused dual effects on the rapidly activating and slowly inactivating K^+ currents in rat artery smooth muscle cells, an increase in current amplitude at concentrations < 0.3 mmol/L and a decrease at > 0.3 mmol/L.

It has been suggested that Kv4.3 and Kv4.2 are the major molecular constituents of native cardiac I_{to} . The ability of nicotine to block Kv4.3 and Kv4.2 might contribute to the previously observed lengthening of cardiac action potential duration in many

preparations (Pappano and Rembush, 1970; Carryl et al., 1992). Nicotine preferentially prolongs initial repolarization and the subsequent plateau phases, consistent with the participation of I_{to} in early phases of repolarization. A-type K^+ channels are not limited to cardiac cells. Both cloned and native A-type currents have been identified in a variety of tissues, including brain and vascular smooth muscle. The A-type K^+ current in vascular smooth muscle is believed to act as a “break” to counteract depolarizing influences that may induce spontaneous action potential activity or oscillatory vasoconstriction. Nicotine is known to have vasoconstriction properties that contribute to the elevation of blood pressure and stroke risks induced by this compound. Nicotine is one of the most potent blockers of A-type channels. 4-aminopyridine (4-AP) is in widespread use as a pharmacological tool for its ability to inhibit A-type K^+ current selectively. However, as documented in many previous reports, several hundred micromolar to several millimolar concentrations of 4-AP are necessary to block Kv4.3 and I_{to} . The potency of nicotine to block Kv4.3 was $\sim 4 \times 10^4$ –fold higher than that of 4-AP ($IC_{50} = 40 \pm 4$ nM for nicotine versus 1.7 ± 0.2 mM for 4-AP). Thus, nicotine can be potentially a useful pharmacological probe to study the role of A-type channels in cardiac electrical activity and the outcome of pharmacological interventions on A-type channels.

Decreases in inward rectifier K^+ currents (Kir) have been implicated in a variety of disease states of the heart, including myocardial infarction and ischemia, cardiac hypertrophy, and heart failure (Beuckelmann et al., 1993). This decrease is often accompanied by cell membrane hyperpolarization and generation of arrhythmias, such as ectopic beats, early afterdepolarization, and triggered activity (Wilde and Aksnes,

1995). A recent work aimed at elucidating the factors involved in action potential initiation in rabbit heart revealed that Kir is a major determinant (Golod et al., 1998). Depression of Kir can lead to increased susceptibility to activation of cells from an ectopic focus, due to increased input impedance and reduced current threshold. Wang et al. (2000a) reported that nicotine could block inward rectifier K^+ channels. It is not unreasonable to speculate that nicotine could worsen the heart diseases by promoting arrhythmias in part by directly blocking cardiac Kir. Simard and Li (2000) demonstrated that nicotine exposure caused a decrease in bioavailability of endogenous NO, as well as block of endogenous NOS activity and oxidative endothelial injury, resulting in a significant increase in Ca^{2+} channel availability. Nicotine increased availability of Ca^{2+} channels and decreased availability of K_{Ca} channels in cerebral arterioles (Gerzanich et al., 2001). Nicotine altered NO signaling of L-type Ca^{2+} channels by a mechanism undescribed, causing blockage of the downregulation of Ca^{2+} channels by NO and cGMP without altering normal upregulation of Ca^{2+} -activated K^+ channels by NO and cGMP. Moreover, Gerzanich et al. (2001) reported the significance of these ion channels by showing reduced pial vasorelaxation in response to NO in animals chronically exposed to nicotine.

In guinea pig ventricular cardiomyocytes, nicotine (100 and 30 μ M) has been shown to inhibit Ca^{2+} and Kv channels (Satoh, 2002). At 1 mM, nicotine blocked Ca^{2+} currents by approximately 90%. The responses to nicotine were not significantly modified by atropine (muscarinic receptor antagonist), hexamethonium (ganglion blocker), and nicotine receptor antagonists (d-tubocurarine and benzoquinonium). Nicotine also strongly inhibited the Kir channels in these cells. The cardiac actions induced by nicotine results from the modulation of ion channels across the cell

membrane. The Kir possessed higher sensitivity to nicotine than Ca^{2+} and Kv channels. In rabbit SA nodal cells with no existence of Kir, nicotine inhibited Ca^{2+} channels but had less or no effect on Kv (Damaj et al., 1993). The Kir mostly contributes to the resting potential, and the Kir inhibition might be expected to lead to partial depolarization of the membrane. However, nicotine did not affect the resting potential in guinea pig ventricular muscles and rabbit SA cells (Satoh, 1997).

1.4.5. Nicotinic acetylcholine receptors

The nicotinic acetylcholine receptor (nAChR) is a ligand-gated channel that delineates a cation-selective pathway across the plasma membrane (Miyazawa et al., 2003). Comprising pentameric combinations of homologous, genetically distinct subunits, nACh receptors are formed from a portfolio of α - and β -subunits ($\alpha 2$ – $\alpha 10$ and $\beta 2$ – $\beta 4$), the differential association of which confers distinct functional and structural properties to the resultant subtypes of nACh receptor (Fenster et al., 1997). nACh receptors mediate fast synaptic transmission in ganglionic neurons. However, there are only a few examples of this in the mammalian CNS (Frazier et al., 1998) where nACh receptors exert a more modulatory influence. In addition to rapid changes in membrane potential, activation of ligand-gated ion channels can also generate longer-lasting effects in the receptive neuron, which contribute to the elaboration of complex intracellular signals that mediate medium- to long-term events. In particular, the role played by intracellular Ca^{2+} signals in the survival of developing neurons, the modulation of their activity and, ultimately, their demise, places Ca^{2+} -permeable nACh receptors in a pivotal position for regulating such events (Role et al., 1996). The structure of the nAChR agonists binding sites has been the subject of intense investigation for more than 20

years. Functional and biochemical data indicate that there are two binding sites for ACh located within the $\alpha_2\beta\gamma\delta$ oligomeric structure of the receptor found in embryonic muscle, and early affinity labeling studies and mutagenesis experiments provided strong evidence that the α subunits play a major role in ligand binding.

Two different types of nicotinic acetylcholine receptors (nAChRs) are distinguished: First, the muscle type confers synaptic transmission at the motor endplate. Second, the neuronal type was originally discovered in the nervous system, but is also expressed in a variety of non-neuronal cells, for example keratinocytes (Grando et al., 1995), bronchial epithelial cells (Conti-Fine et al., 2000) and the epithelium of the gastrointestinal tract (Wessler et al., 1998). In these tissues nAChRs participate in the regulation of a broad spectrum of cellular functions, for example ciliary motility, formation and maintenance of cell-cell contacts, resorption and secretion (Wessler et al., 1998).

In view of the profound pathogenic effects of chronic nicotine exposure on vascular wall structure, the presence of nAChRs shall be expected in the vascular wall. Their occurrence on nerve fibres innervating blood vessels is well established. Prejunctional nAChRs on sympathetic axons inhibit noradrenaline release (Edvinsson et al., 1977), and stimulation of nAChRs located on nitrergic parasympathetic axons leads to release of NO with subsequent vasodilatation (Ayajiki et al., 1994). There is, however, growing evidence for an additional non-neuronal localisation of nAChRs in the arterial wall. Nicotine induces vasoconstriction in the denervated pulmonary circulation in the dog (Samanek and Aviado 1966), it relaxes the isolated rat renal artery (Emre et al., 1999) and it impairs endothelial-dependent relaxation in the hamster cheek pouch (Mayhan and Patel 1997). In humans, Stefanadis et al. (1997) showed an

ultrasonically measurable decrease of aortic elasticity while patients were smoking. Primary cell cultures of arterial endothelial cells (ECs) express functional nAChRs (Conti-Fine et al., 2000; Wang et al., 2001), and *in vivo* ligand binding studies showed specific binding sites for [³H]-nicotine in human cerebral and pial vessels (Kalaria et al. 1994). An immunohistochemical study of the fetal monkey lung localised the $\alpha 7$ -subunit to the arterial wall (Sekhon et al., 1999), but data on the detailed distribution of the individual nAChR subunits in the arterial wall and arterial system, however, are still lacking.

Brüggmann and colleagues (2002) reported that nAChR subtypes are expressed by both ECs and vascular SMCs in rat arteries *in situ*. Consequently, these main cellular components of the vascular wall shall be considered as direct targets of nicotine and endogenous nAChR ligands such as acetylcholine. The large conductive arteries investigated in that study are devoid of a cholinergic innervation, so that an endogenous ligand addressing their nAChRs will not be derived from a neuronal source. Instead, ECs have been recognized as a major source of ACh. They express the acetylcholine-synthesizing enzyme, choline acetyltransferase, and they synthesize and release ACh (Haberberger et al., 2000; Kawashima et al., 1990). Thus, there is evidence for a locally acting autocrine and paracrine non-neuronal cholinergic system in the arterial wall (Kummer and Haberberger 1999) that includes nAChRs. Well in line with this notion, a nicotinic angiogenic pathway has recently been described that operates both *in situ* in a disc angiogenesis system and *in vivo* in plaque formation during atherosclerosis, tumour growth and wound healing (Heeschen et al., 2001). This system acts endogenously since angiogenesis in a murine hind limb ischaemia model is significantly reduced by the non-selective nAChR antagonists, mecamylamine (Heeschen et al., 2002).

Vascular SMCs cultured from the rat tail artery are likely to express functional nAChRs since they respond to nicotine with a membrane depolarisation and altered K^+ currents (Tang et al., 1999; Wang and Wang 2000). The subunits involved in these effects have not been identified. Brüggmann and colleagues (2002) identified $\alpha 3$ -, $\alpha 5$ -, $\alpha 7$ - and $\alpha 10$ -subunits in vascular SMCs while the distribution of the subunits $\alpha 2$, $\alpha 4$ and $\alpha 6$ was more selective. The subunit $\alpha 2$ was detected in proximal elastic arteries, while $\alpha 4$ and $\alpha 6$ occurred only distal to the ascending aorta. The parallel occurrence of $\alpha 4$ and $\alpha 6$ is consistent with the observation that $\alpha 4$, $\alpha 6$ and $\alpha 2$ are able to build up a functional nicotinic receptor (Kuryatov et al., 2000).

The occurrence of the $\alpha 10$ -subunit in vascular SMCs without the simultaneous expression of the $\alpha 9$ -subunit is noteworthy because $\alpha 9$ is its only known interacting partner to form a functional nAChR (Elgoyhen et al., 2001). Accordingly, cell types expressing the $\alpha 10$ -subunit have so far always been found to coexpress the $\alpha 9$ -subunit (Elgoyhen et al., 2001). While coexpression studies of subunits $\alpha 2$, $\alpha 4$, and $\alpha 6$ with $\alpha 10$ have been conducted and did not result in a functional receptor, the properties of an $\alpha 7/\alpha 10$ combination have not been reported yet (Elgoyhen et al., 2001). The extensive codistribution of $\alpha 7$ - and $\alpha 10$ -subunits in vascular SMCs of rat arteries may be taken as a hint to a functional coupling of these subunits.

A striking feature of the distribution pattern of nAChRs in the rat arterial system is its extensive heterogeneity. While every α -subunit except $\alpha 9$ was detected in the thoracic aorta, intrapulmonary arterial branches contained only $\alpha 7$ immunoreactivity, and other vascular beds held intermediate positions with respect to the extent of α -subunit expression. Current knowledge does not allow correlating these distribution patterns to specific functions, but it can be anticipated that at least some components of

nAChR-mediated signaling in the arterial wall are highly specific for individual arteries. One of the implications derived from this may be different vulnerabilities of individual arterial beds to externally applied nicotine.

1.5. OXYGEN FREE RADICALS

1.5.1. Definition of oxygen free radicals

Oxygen free radicals have been implicated in the pathophysiology of various disease processes including ischemia-reperfusion injury, heart failure, hypercholesteremic atherosclerosis, diabetes mellitus, hemorrhagic and endotoxic shock, and peripheral vascular disease (Hogg 1998; Bergamini et al., 2004). Free radicals are defined as molecules that contain an unpaired electron. Organic molecules normally possess an even number of electrons with each orbital being occupied by two electrons having an opposite magnetic moment and spin. Consequently, free radicals possess such a great chemical reactivity that rate constants can reach the limit for free diffusion ($10^9 \text{M}^{-1}\text{s}^{-1}$). Generally, the simpler the structure, the shorter the life of free radicals. Complex molecules, like triphenylmethyl, have more stability and a longer life span. This means that, in effect, these reactions proceed nearly instantaneously.

It is interesting to note that much of the recent research into free radical reactions is a consequence of the Cold War (Olinescu and Smith, 2002). Following the invention of nuclear weapons in the Second World War, there was a great deal of interest in radiation disease. The discovery that free radicals are involved in the first steps of the consequences of irradiation of living organisms attracted much attention to the field of

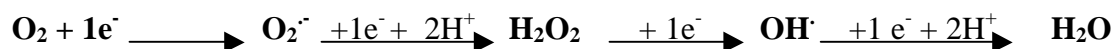
free radical research. Likewise, the interest in free radicals by physicians and medical researchers is based on their role in physiology and pathophysiology.

1.5.2. Generation of oxygen free radicals

Oxygen-derived free radicals include the superoxide anion ($O_2^{\cdot-}$), and hydroxyl ($\cdot OH$), peroxy (ROO^{\cdot}), alkoxy (RO^{\cdot}), and hydroxyperoxy (HOO^{\cdot}) radicals. Oxygen metabolites that contain an even number of electrons, such as H_2O_2 and hypochlorous acid ($HOCl$), are not oxygen-derived free radicals (Bergamini et al., 2004).

1.5.2.1. Superoxide anion ($O_2^{\cdot-}$)

Univalent reduction of molecular oxygen generates $O_2^{\cdot-}$, which is very unstable. It can act as both oxidant and reductant (Wu et al., 2001). It oxidizes ascorbate, sulfhydryl-containing compounds, sulfate, and catecholamines. It reduces ferric iron and quinines. It inactivates a variety of enzymes (e.g., tRNAase, RNAase, glyceraldehydes-3-phosphatedehydrogenase, and aconitase). $O_2^{\cdot-}$ is not highly toxic, and most of the damaging effects are due to highly toxic $\cdot OH$. Oxygen is metabolized as shown in Fig. 1.6. The sequential transfer of one electron to oxygen (i.e. the sequential reduction of oxygen) results in the formation of two intermediates (less reactive $O_2^{\cdot-}$ and highly reactive hydroxyl radical $\cdot OH$), a relatively stable and long-lived intermediate (hydrogen peroxide, H_2O_2), and a stable and harmless end product (water).



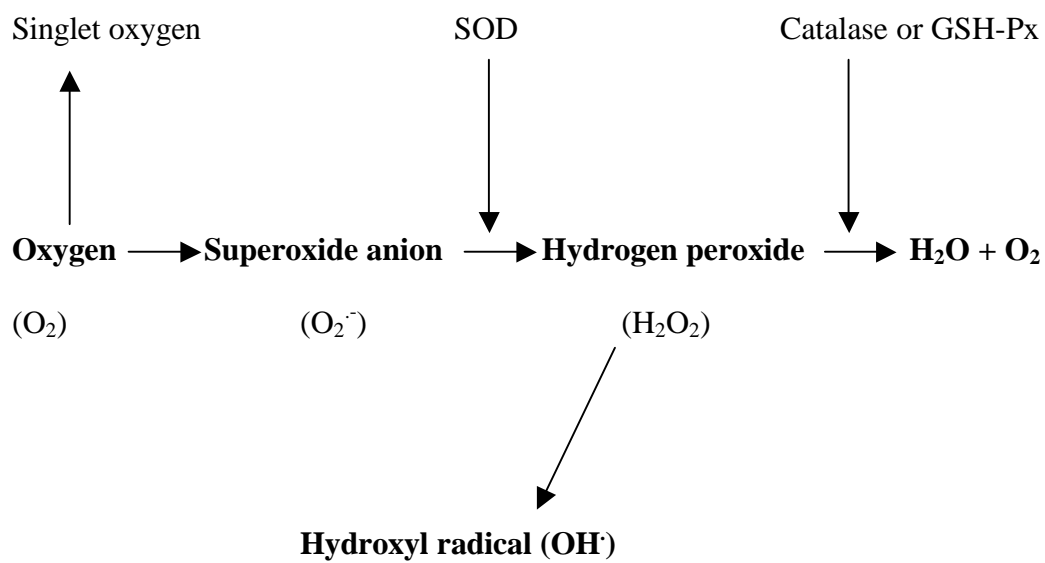


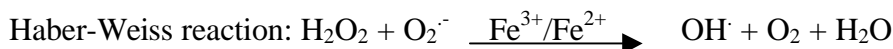
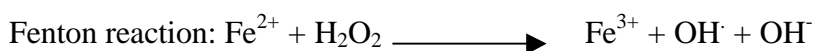
Figure 1.6. Schematic diagram of oxygen free radicals generation. SOD indicates superoxide dismutase; GSH-Px, glutathione peroxidase (Prasad, 2000).

1.5.2.2. Hydrogen Peroxide (H₂O₂)

H₂O₂ is formed by dismutation of O₂^{•-}, a process accelerated by SOD (Bergamini et al., 2004). It is a relatively stable oxidant and very lipophilic and hence crosses cell membranes rapidly. It can inactivate some enzymes by oxidizing their reactive sulfhydryl groups. Catalase and glutathione peroxidase (GSH-Px) convert it to H₂O + O₂.

1.5.2.3. Hydroxyl radical (OH[•])

Hydroxyl radical is generated in two reactions.



A two-step reaction is involved in Haber-Weiss reaction, in which OH[•] is formed in a traditional Fenton reaction followed by reduction of the iron by superoxide anion (Fe³⁺ + O₂^{•-} → Fe²⁺ + O₂). In this sequence, recycling of the metal catalyst maintains a reaction chain.

1.5.2.4. Alkoxy radicals (OR[•]) and peroxy radicals (OOR[•])

Lipid peroxidation generates two additional reactive and short-lived radicals, namely, alkoxy radicals (OR[•]) and peroxy radicals (OOR[•]). In addition, organic peroxides (lipid hydroperoxides, ROOH) are also formed as relatively more-stable intermediates or end products.

1.5.2.5. Nitric oxide (NO·)

NO· is also an ROS because it has an unpaired electron associated with the oxygen atom. An interaction of NO· with superoxide anion produces a labile non-radical compound, the peroxynitrite anion ($O_2^{\cdot -} + NO^{\cdot} \longrightarrow ONOO^{\cdot -}$). When peroxynitrous acid is formed by acidification of the anion ($ONOO^{\cdot -} + H^+ \longrightarrow ONOOH$), it will spontaneously decompose by homolytic scission to yield the highly reactive hydroxyl radical ($ONOOH \longrightarrow OH^{\cdot} + ONO^{\cdot}$).

1.5.2.6. Oxidized glutathione (GSSG)

GSSG, a disulfide-linked homodimer, is known for its action to oxidize protein thiols. Either an intramolecular disulfide bond between adjacent sulfhydryls in the protein or a mixed disulfide bond between the protein and glutathione will be formed as the result of the GSSG-induced oxidation. Being generated via the action of glutathione peroxidase (GSH-Px), GSSG is reduced back to glutathione (GSH) via the activity of GSH reductase (GSH-Rt), which serves to maintain GSSG levels between one-tenth and one hundredth those of total GSH. GSH, a tripeptide, is the single most abundant redox-active sulfhydryl compound within the cytosol of mammalian cells. By providing reducing equivalents for the redox reaction catalyzed by GSH peroxidase, GSH represents the major antioxidant defense mechanism.

1.5.3. Sources of oxygen free radicals

Normal cellular energy metabolism is not 100% efficient, and it is considered that between 1% and 5% of all oxygen atoms used in energy metabolism escapes as oxygen free radicals. Oxidative reactions are also very important in other biological

reactions and many of these have the potential to generate oxygen free radicals under physiological conditions. For example, both cytochrome P450s and cyclooxygenase generate oxygen free radicals. Thus, the production of oxygen free radicals can be a natural part of cellular function. Two main endogenous $O_2^{\cdot -}$ generating enzyme systems are xanthine oxidase (XO) and NADH/NADPH oxidase. *In vivo*, xanthine dehydrogenase is converted to XO which mediates the metabolism of nucleotides such as xanthine and hypoxanthine (HX) to form $O_2^{\cdot -}$, H_2O_2 , and uric acid (Fig. 1.7). Inhibitors of XO, e.g. allopurinol and oxypurinol, have been used to attenuate the injury process and have permitted the identification of XO as a mediator of tissue injury. NADH/NADPH oxidase is a multi-subunit enzyme, which has been claimed to be the major source of $O_2^{\cdot -}$ production in many tissues.

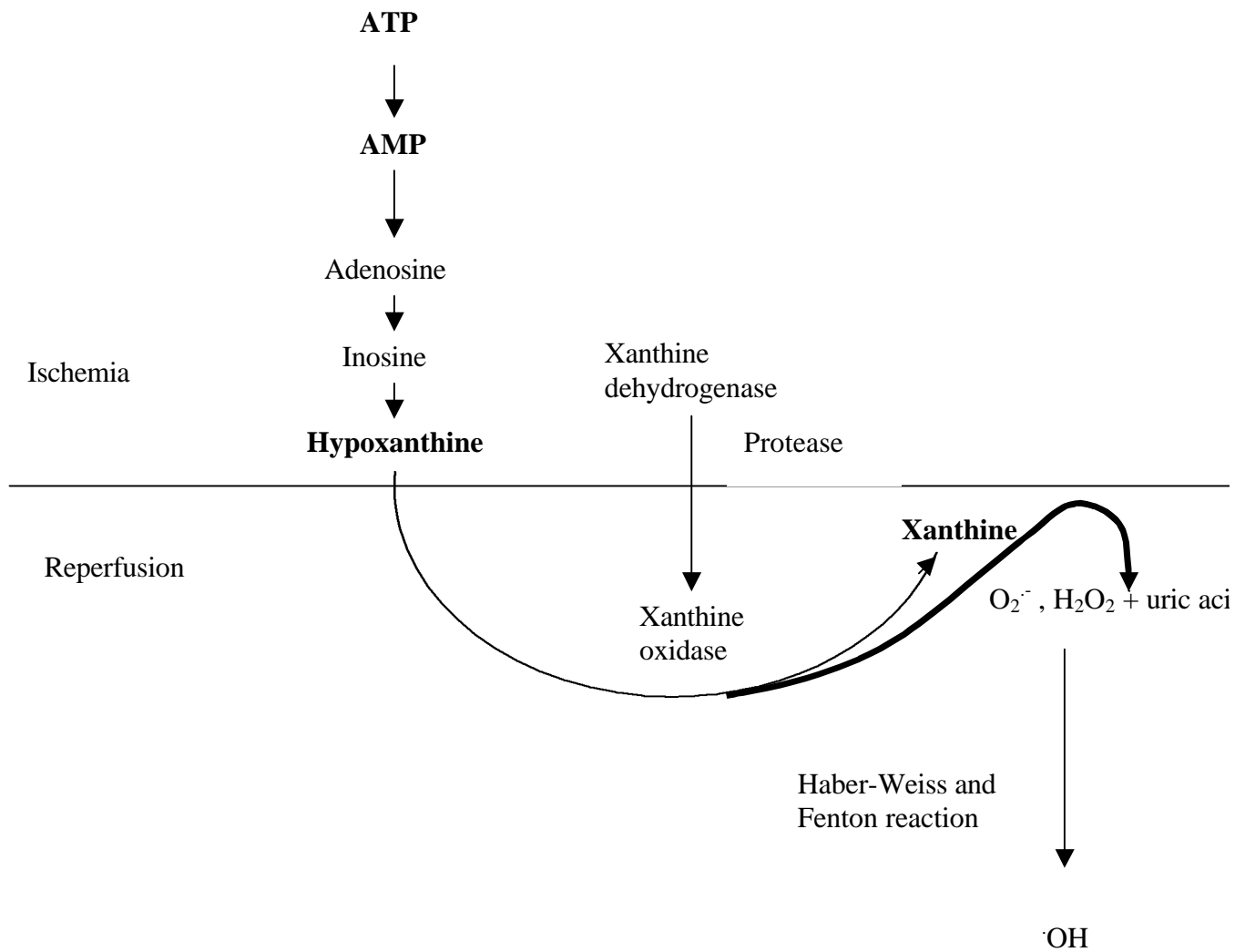


Figure 1.7. Schematic diagram for the formation of oxygen radicals with interaction of an adenine nucleotide and xanthine oxidase. ATP indicates adenosine triphosphate, AMP, adenosine monophosphate (Prasad, 2000).

1.5.4. Antioxidants

Antioxidants, when present at a low concentration compared with the concentration of an oxidizable substrate, significantly delay or inhibit oxidation of that substrate. The body is equipped with a variety of antioxidants to protect against excessive radical generation and the consequences thereof. Intracellular antioxidants scavenge aberrant free radicals locally. Extracellular antioxidants break the chain of radical reaction propagation and remove metal ions and heme proteins by sequestering them and making them incapable of generating free radicals. There are two classes of antioxidants: enzymatic and nonenzymatic, which includes exogenous and endogenous substances. Enzymatic antioxidants include: SOD, catalase, GSH-Px, and other peroxidases. Nonenzymatic antioxidants include those that are water soluble (vitamin C, uric acid, glucose, pyruvate, bilirubin, and sulfhydryl groups), lipid soluble (alpha tocopherol, beta carotene, ubiquinol 10), and plasma protein-bound (transferrin, albumin, ceruloplasmin, haptoglobin, and hemopexin molecules).

Antioxidants are substances that donate an electron to a free radical thus inactivating the radical species. Some of these antioxidants exist in several forms. Membrane, cytosolic and plasma isoforms of GSH-Px have been reported. Similarly, there are mitochondrial, cytosolic, and extracellular isoforms of SOD. The vast network of intracellular and extracellular antioxidants strongly suggests that the levels of endogenous antioxidants must be tightly regulated for cell survival.

1.6. ACETYLCHOLINE (ACh)

1.6.1. Acetylcholine and neurotransmission

ACh is best known as a neurotransmitter. However, ACh probably evolved long before the appearance of the nervous system, since the machinery to synthesize and degrade ACh is present in bacteria, fungi, protozoa and plants. Even in so-called higher organisms, ACh is present in nonneuronal tissues such as the placenta, suggesting that ACh has cellular functions other than neurotransmission.

As a neurotransmitter, ACh was among the first to be identified. ACh was identified as a possible mediator of cellular function by Hunt in 1907. In the 1910s, it was shown by Sir Henry Dale to mimic the effect of parasympathetic nerve stimulation and to have different actions depending on the tissue. The latter observation suggested the presence of separate 'receptive substances'. ACh release upon nerve stimulation was demonstrated in the 1920s by Otto Loewi, in elegantly simple experiments using two frog hearts. Despite this impressive beginning, a definitive mapping of central cholinergic pathways was not available until the 1960s, when Shute and Lewis published a series of studies using acetylcholinesterase (AChE), providing the first overall picture of cholinergic pathways (Lewis and Shute, 1967; Shute and Lewis, 1967). However, the results of these landmark studies remained somewhat tentative, because AChE is present not only in cholinergic neurons, but also in noncholinergic neurons such as certain noradrenergic and dopaminergic neurons. In the early 1980s, antibodies to choline acetyltransferase (ChAT), the biosynthetic enzyme for ACh, became available (Kimura et al., 1981; Crawford et al., 1982) and these antibodies made it possible, for the first time, to identify cholinergic pathways definitively.

The role of ACh as a key neurotransmitter in the central and peripheral nervous system is well established. However, the role of ACh may be broader because ACh may also function as an autocrine or paracrine signaling molecule in a variety of nonneuronal tissues (Wessler et al., 1998). ACh is the first neurotransmitter whose diffusion from the central nervous system was investigated and whose extracellular level variations were correlated to changes in neuronal activity. This was done initially by means of the cup technique and then by the microdialysis technique (Metzen et al., 2003). The latter, notwithstanding some technical limitations, makes it possible to detect variations in extracellular levels of ACh in unrestrained, behaving animals. Investigations had shown that there are changes in ACh release during performance of operant tasks, exposure to novel stimuli, locomotor activity, and the performance of spatial memory tasks, working memory, and place preference memory tasks. Activation of the forebrain cholinergic system has been demonstrated in many tasks and conditions in which the environment requires the animal to analyze novel stimuli that may represent a threat or offer a reward. The sustained cholinergic activation, demonstrated by high levels of extracellular ACh observed during the behavioral paradigms, indicates that many behaviors occur within or require the facilitation provided by the cholinergic system to the operation of pertinent neuronal pathways (Wessler et al., 1999).

1.6.2. The non-neuronal cholinergic system in the endothelium

It is becoming increasingly apparent that ACh is not used exclusively by the nervous system. Thus the non-neuronal ACh has been found not only in the major classes of the animal kingdom, but also in primitive plants. This has led to the concept of “non-neuronal cholinergic system” (Wessler et al., 1999), the essential elements of

which have been demonstrated in various human tissues, including immunocompetent cells such as lymphocytes, respiratory epithelial cells and keratinocytes. It is well known that ACh mediates multiple effects on the vascular endothelium, for example the generation of the endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980). Therefore it appears of great biological interest whether endothelial cells are endowed with the biochemical apparatus to synthesize, transport, store and secrete ACh by themselves to control endothelial cell functions by local regulatory loops. Parnavelas and co-workers (1985) used ultrastructural immunocytochemistry to localize the synthesizing enzyme ChAT in endothelial cells of rat brain capillaries. These authors suggested that the release of ACh from endothelial cells as a result of ischemic damage could be protective against further hypoxia via the vasodilatory activity of ACh. Following isolation of rat brain cortex capillaries, a ChAT specific activity of 0.264 nmol/mg protein/min in the dissociated endothelial cells has been reported (Gonzalez and Santos-Benito, 1987). However, in human fetal brain microvessels in culture, other authors failed to detect ChAT activity (Kasa et al., 1991). A very significant finding was provided by Kawashima and colleagues (1990), who studied ACh synthesis and release in bovine carotid artery endothelial cells *in vitro*. Analytical measurement by radioimmunoassay and HPLC indicated that the endothelial cells synthesize ACh and that the synthesized ACh was rapidly released from the cells (Kawashima et al., 1990).

In endothelial cells isolated from porcine cerebral microvessels, both HPLC and radioimmunoassay methods were used to measure ACh content (Ikeda et al., 1994). Phorbol ester was found to up-regulate ACh synthesis via a protein kinase C-independent pathway (Ikeda et al., 1994). However, studies with the ChAT inhibitor, bromoacetylcholine, suggested that ACh synthesis occurred independently of ChAT;

i.e., an enzyme different from ChAT mediated the synthesis of ACh. Further studies by the same group investigated choline uptake in porcine brain endothelial cells and its utilization for both phospholipid and ACh synthesis (Suzuki et al., 1996b). Recently, an immortalized rat brain endothelial cells (RBE4) have been used to engineer stable endothelial cell lines producing ACh (Malo et al., 1999). This has been achieved by employing an expression vector construct to transfect ChAT. Further studies have been carried out on endothelial cells transfected with the vesicular ACh transporter (VACHT) gene. It is to be expected that such genetically modified endothelial cell types may help unravel some of the regulatory pathways involved in ACh synthesis, storage, transport and release. The ability of the endothelium to respond to ACh has been well documented. This response depends on the expression of ACh receptors (AChR), which are of either muscarinic (mAChR) or nicotinic (nAChR) type. The pioneering work of Furchgott and Zawadzki (Furchgott and Zawadzki, 1980) demonstrated that following interaction of ACh and mAChR, the endothelium produced a vasodilator, EDRF, now accepted as nitric oxide. Utilizing an *in situ* hybridization technique, it was demonstrated previously that cultured endothelial cells from the human aorta express nAChR of the $\alpha 3$, $\alpha 5$, $\beta 2$ and $\beta 4$ subtypes (Macklin et al., 1998). Patch clamp experiments confirmed the functionality of these receptors. On the endothelium of human pulmonary veins, M1 muscarinic receptors have been demonstrated (Walch et al., 2000). Offner and colleagues' (1992) work has been focused on the use of human endothelial cell culture systems to investigate the pathobiology of the endothelium in various disease states with special emphasis on inflammation, sepsis and tumor extravasation. Using monolayer cultures of human umbilical vein endothelial cells, HPLC techniques were used to

demonstrate the production of ACh and a closely related compound. Similar results were found from a human angiosarcoma cell line. The non-neuronal cholinergic system in the endothelium represents the standing at the beginning of this fascinating chapter. There are many unanswered questions. One of the important tasks is to further define the subcellular localization of the synthesizing enzyme ChAT, as well as the vesicular transporter VACHT. The indications from the immunoelectron microscopic study that VACHT can be detected in vesicles of the endothelial cytoplasm and the cytoskeletal-like distribution demonstrated by light microscopic immunochemistry requires further investigation. The central question in this field of research must however remain the delineation of the functional significance of endogenous endothelial ACh. Thus, the possible role in endothelial cytokine expression, induction and regulation of cell adhesion molecules, proliferation, angiogenesis and hemostatic control should be advanced during the next years.

1.6.3. Acetylcholine and ion channels

ACh is well known as a key-neurotransmitter of the parasympathetic nervous system. To understand its role it is better to study the heart as an example of ACh effect. Autonomic regulation of the heart is effected mainly by two systems: the sympathetic and parasympathetic nervous systems. In the resting heart, the major influence is parasympathetic, whereas during exercise the situation is reversed and sympathetic stimulation becomes predominant. The sympathetic and parasympathetic subdivisions of the autonomic nervous system can be viewed, in general terms, as opposing each other (Yin-Yang) (Fig. 1.8). For example, sympathetic stimulation increases heart rate, blood pressure, and myocardial contractility, whereas parasympathetic stimulation slows the

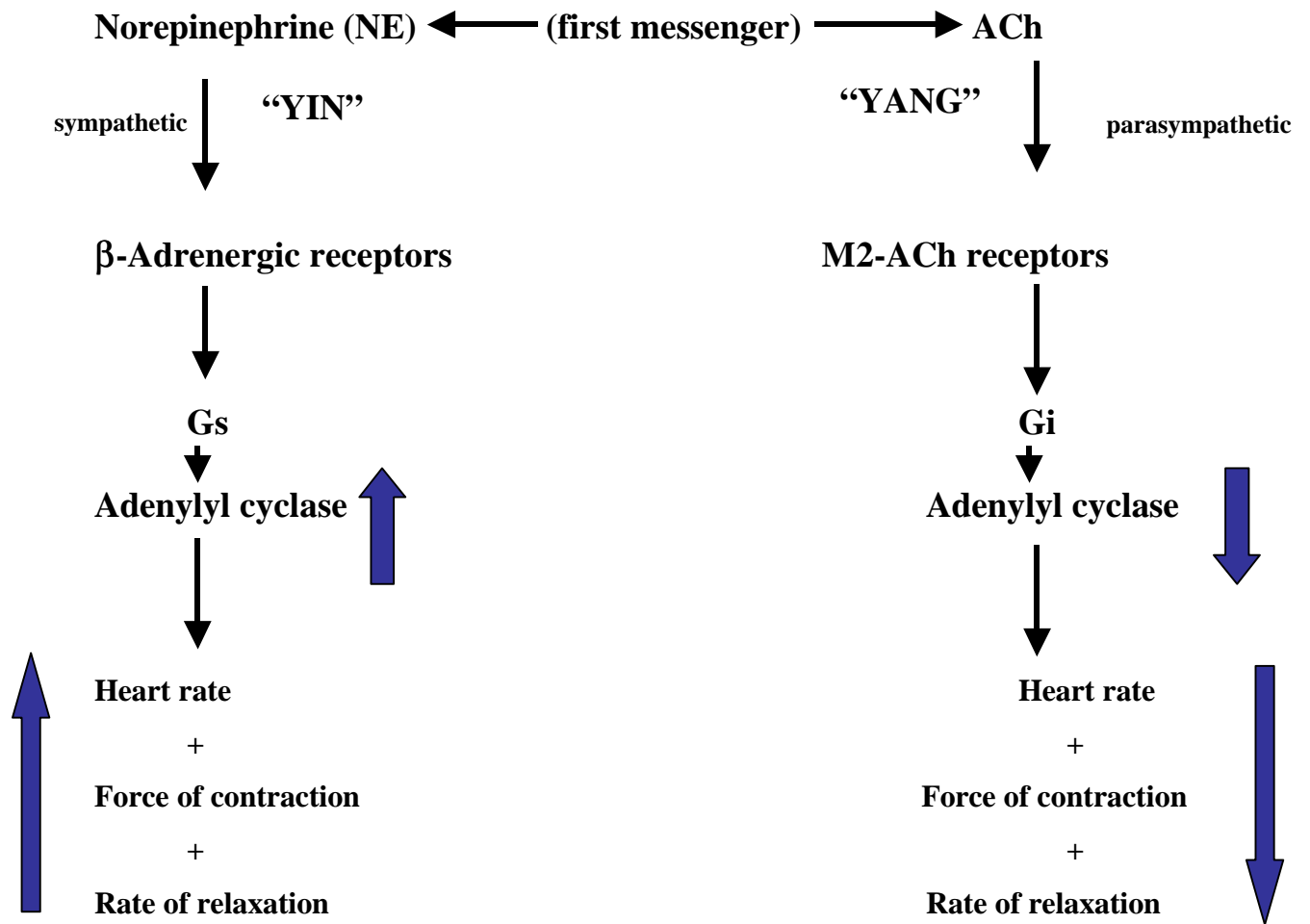


Figure 1.8. Extrinsic regulation of the heart.

heart, decreases blood pressure, and causes a marked negative inotropic effect on the atria (Loffelholz and Pappano, 1985). Until recently, it was generally taught that the ventricles were insensitive to both parasympathetic stimulation and ACh; however, it is now apparent that cholinergic nerves supply the ventricles and conducting tissues of the His-Purkinje system. The inhibitory effects of cholinergic stimulation on the ventricles and His-Purkinje system are much more prominent following β -adrenergic receptor stimulation, so that parasympathetic stimulation blunts the response to sympathetic stimulation. There are two important types of parasympathetic (cholinergic) receptors: the nicotinic receptors, which are found mainly in skeletal muscle and ganglial cells, and the muscarinic receptors. Muscarinic receptors are encoded by a number of different genes; most important are the M1-subtype, found mainly in autonomic ganglia and the central nervous system, the M2-subtype in the heart, and the M3-subtype in smooth muscle and secretory cells. All are blocked by atropine.

Many clinical examples of sinus bradycardia result from excessive parasympathetic (vagal) tone, especially in younger patients. Chronic slowing of the sinus pacemaker is regularly seen in “athlete’s heart,” whereas sudden sinus bradycardia can occur abruptly in vasovagal syncope, the old-fashioned “swoon”. Parasympathetic slowing of the sinus pacemaker is mediated by changes in at least three different ion channels (Pappano and Mubagwa, 1991); these changes are initiated when acetylcholine binds to cardiac M2 muscarinic receptors (Fig. 1.9). When ACh binds to the muscarinic receptors it causes conformational changes of those receptors causing them to bind to the G proteins in the plasma membrane. The G proteins are heterotrimers made up of three subunits: $G\alpha$, $G\beta$, and $G\gamma$. The larger $G\alpha$ subunits are the major participants in the

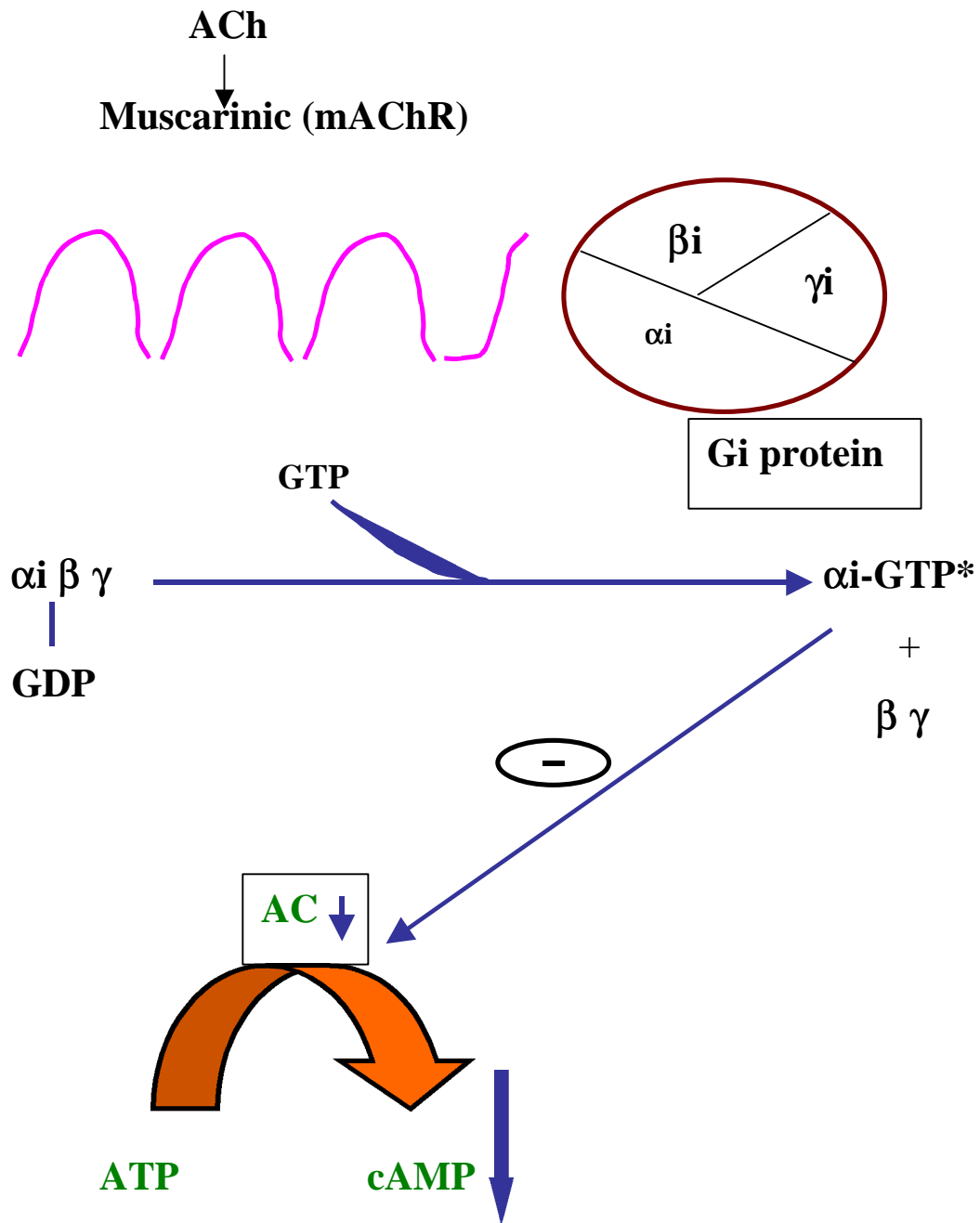


Figure 1.9. Schematic diagram of ACh binding to cardiac M2 muscarinic receptors.

function of the G proteins; they are also largely responsible for the specificity of the G proteins. Interaction of G_i -GDP and promotion of GDP-GTP exchange on α unit of the G_i protein follows. Interaction between α_i -GTP and adenylyl cyclase results in an inhibition of adenylyl cyclase. Inhibition of the adenylyl cyclase causes decrease of the cyclic-AMP levels and therefore, decreases of the heart rate and the inotropic activity of the heart.

The first mechanism by which ACh and other muscarinic agonists cause sinus bradycardia is by hyperpolarizing the SA node, which occurs when either ACh or adenosine activates ACh-activated potassium current ($I_{k,ACh}$) in the SA node (Fig. 1.10). The effects of M2 receptors on $I_{k,ACh}$ appear to be mediated by an inhibitory G_i that can be activated when ACh binds to its receptor. The ligand-receptor complex, by activating this G-protein, opens channels that carry the potassium current $I_{k,ACh}$. This brings resting potential closer to the Nernst potential for potassium. The ability of $I_{k,ACh}$ to hyperpolarize the resting cell reflects the fact that the diastolic potentials in the SA node are considerably lower than equilibrium potential for K^+ (E_K). $I_{k,ACh}$ is a G-protein-gated inwardly rectifying K^+ channel (GIRK). The GIRK channel class comprises four members: GIRK1, GIRK2, GIRK3 and GIRK4. The cardiac $I_{k,ACh}$ is a heterotetramer consisting of two GIRK1 and two GIRK4 subunits.

The second ion channel that slows the SA node in response to ACh carries the inward pacemaker current, funny current (I_f). This pacemaker current is inhibited by a direct effect of G_i and an indirect effect that occurs when G_i inhibits cyclic AMP (c-AMP) production. The decrease in cAMP concentrations also attenuates the calcium

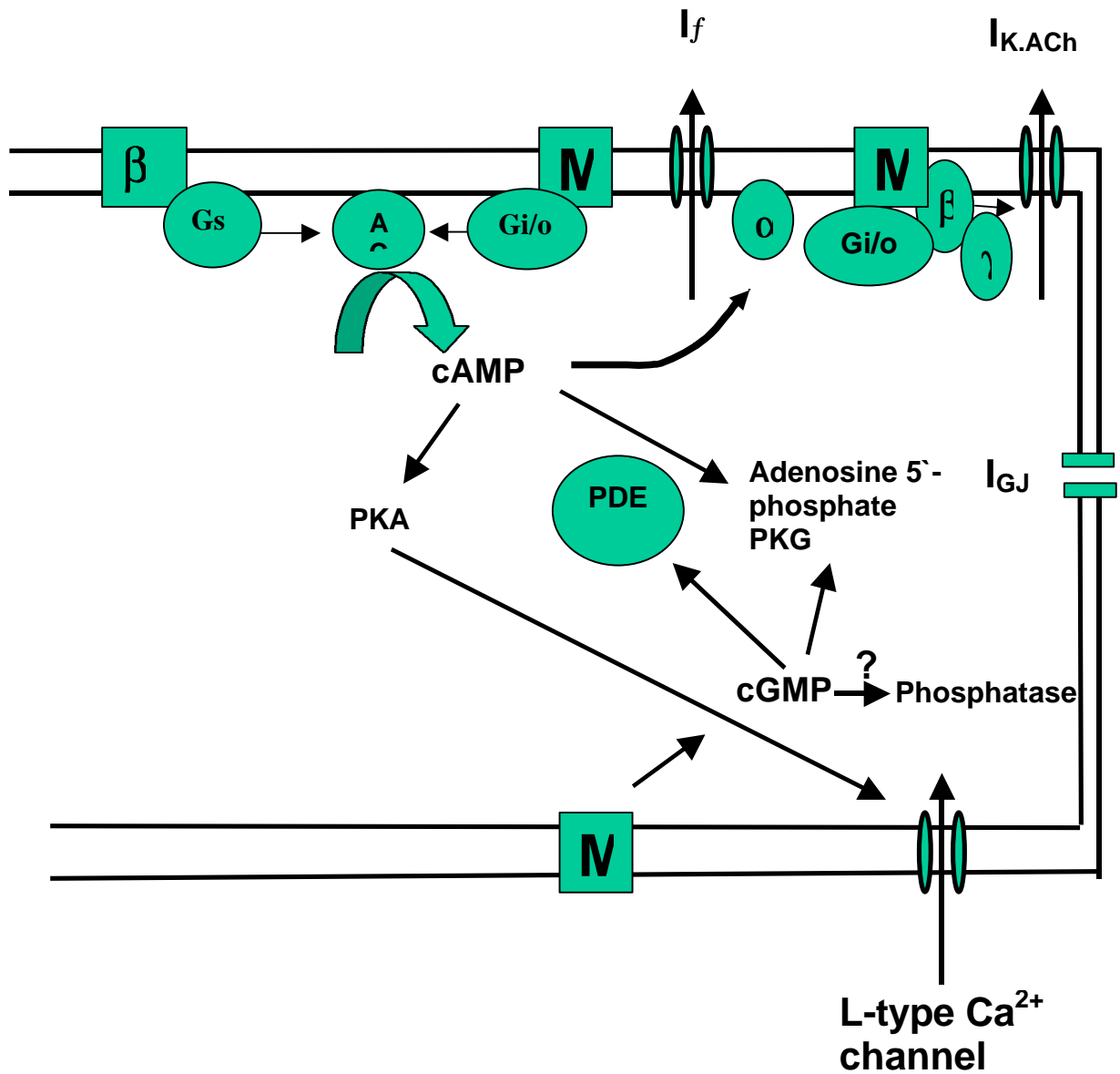


Figure 1.10. Overview of the main ion channels in the heart affected by stimulation of muscarinic receptors at physiological concentrations. Abbreviations: *I_f*, pacemaker current; *I_{K,ACh}*, ACh-sensitive K⁺ current; *I_{GJ}*, gap junctional current; M, Muscarinic receptor; β, β-adrenoceptor; AC, adenylyl cyclase; PDE, phosphodiesterase; PKG, cGMP-dependent protein kinase; PKA, cAMP-dependent protein kinase.

current I_{Ca} by reducing the probability of calcium channel opening. The resulting inhibition of I_{Ca} by reducing the major inward current that depolarizes the SA node also contributes to the SA block sometimes caused by vagal stimulation.

If ACh is applied to atrial, Purkinje or nodal cells, hyperpolarization of the membrane typically occurs, which has been ascribed to the opening of $I_{k,ACh}$. The effects of mAChR receptor stimulation on $I_{k,ACh}$ are transduced via the pertussis-toxin-sensitive Gi protein (Sakmann et al., 1983). For this action Mg^{2+} and GTP are required (Kurachi et al., 1986a; Kurachi et al., 1986b). The effect of muscarinic agonists can be mimicked with stable GTP analogues. If the cells are exposed to sufficient concentrations of muscarinic agonists continuously, a decrease in the current response has been detected without a change in the shape of the current-voltage relationship (Carmeliet and Mubagwa, 1986).

The opening of $I_{k,ACh}$ in pacemaker cells starts with a latency of 30 to 100 ms (Hartzell, 1980), which is longer than that for ligand-gated ion channels, but is considerably faster than that of second-messenger-regulated systems such as the β -adrenoceptor-cAMP- I_{Ca} system (Yatani and Brown, 1989). In order to elucidate the underlying mechanisms, Soejima and Noma (1984) carried out a series of very elegant patch-clamp experiments on rabbit atrial cells: an on-cell patch in the absence of ACh revealed occasional opening of K^+ channel that was not altered if ACh was added to the bath solution. However, when ACh was perfused into the pipette, the authors observed a dramatic increase in the opening of $I_{k,ACh}$. These experiments were consistent with the view that the channel and receptor are located in close proximity in the patch. In contrast, a second-messenger ion channel system (such as β -adrenoceptor-cAMP- I_{Ca}

system) would (due to the diffusibility of the second messenger) respond to the addition of the agonist to the bath in an on-cell patch situation with channel openings throughout the cell. Using excised patch experiments it has been shown that the exposure of the inner face of the membrane to previously activated Gi protein results in opening of $I_{k,ACh}$ (Yatani et al., 1988). From the above experiments it was concluded that $I_{k,ACh}$ is not activated by a cytosolic second messenger, but that the activating system is membrane delimited and can not spread over the cell.

Subsequently, interest was focused on the question of whether the α - or the $\beta\gamma$ -subunit of Gi protein activates the channel. While the first investigations suggested the α -subunit of Gi to activate $I_{k,ACh}$ (Yatani et al., 1987), others favored the $\beta\gamma$ -subunit as activator (Logothetis et al., 1987). In addition the $\beta\gamma$ -subunit was supposed to regulate channel opening indirectly via phospholipase A2 (PLA2) activation and subsequent release of arachidonic acid (Kurachi et al., 1989). Prolonged (≥ 2 min) M2-cholinoceptor activation can result in an inhibition of $I_{k,ACh}$ in a pertussis toxin-insensitive manner (Bunemann et al., 2000). This desensitization of the channels did not require phosphorylation or internalization of the M2-ChR. It was concluded that prolonged M2 and M4 cholinoceptor stimulation can inhibit $\beta\gamma$ -subunit-activated $I_{k,ACh}$ involving non-pertussis toxin sensitive G protein.

In human heart, $I_{k,ACh}$ has been identified in the atrium (Heidbuchel et al., 1990) as well as in the ventricle (Koumi and Wasserstrom, 1994). In both tissues $I_{k,ACh}$ was directly activated by ACh. While the channel kinetics and characteristics were similar in atrium and ventricle, the two tissues differed with regard to the ED_{50} of the ACh-induced activation of $I_{k,ACh}$: in atrium half maximum activation was achieved with $0.03 \mu M$ ACh,

whereas in the ventricle EC_{50} was $0.13 \mu M$. In both tissues $I_{k,ACh}$ was activated via Gi protein (Koumi and Wasserstrom, 1994). However this direct $I_{k,ACh}$ activation in human ventricular tissue does not seem to contribute substantially to inotropy since a direct negative inotropic effect of muscarinic stimulation is lacking from the human heart. There is no evidence for an inhibition of the transient type Ca^{2+} channels by muscarinic agonists including ACh (Cerbai et al., 1988). In contrast, L-type Ca^{2+} channel is inhibited by muscarinic stimulation in both atrium and ventricle. However, while in atrium no prior elevation of intracellular cAMP concentration is required, in ventricle the inhibitory effect on the L-type Ca^{2+} channel is typically seen only if cAMP has been elevated, which has been defined as ‘accentuated antagonism’ (Levy, 1971). In atrial cells a concentration-dependent inhibition of L-type Ca^{2+} channels up to 35 % following stimulation with muscarinic agonists or adenosine can be observed if $I_{k,ACh}$ is blocked by cesium ions otherwise the activation of $I_{k,ACh}$ would shorten the time window for L-type Ca^{2+} channel (Cerbai et al., 1988). However, according to these authors the activation of $I_{k,ACh}$ is the dominant effect over L-type Ca^{2+} channels inhibition in the atrium. The underlying mechanism does not require enhanced cAMP levels and, thus, is not yet well understood.

Besides this ‘direct’ effect on the L-type Ca^{2+} channel seen only in atrium, an ‘indirect’ inhibition of L-type Ca^{2+} channel can be observed after muscarinic stimulation. The ‘classical’ view of the underlying mechanism is that muscarinic stimulation leads via pertussis toxin sensitive Gi protein to an inhibition of adenylyl cyclase, thereby decreasing cAMP levels, thus leading to reduction of the previously augmented L-type Ca^{2+} channel (augmentation by isoprenaline, forskolin or

isobutylmethoxyxanthine in frog and guinea pig ventricle) (Fischmeister and Hartzell, 1986). L-type Ca^{2+} channels that have been stimulated with cAMP directly or with cAMP-dependent protein kinase can not be inhibited with muscarinic agonists, so that inhibition of formation of cAMP seems to be the obvious explanation (Hescheler et al., 1986). As reviewed by Reuter (Reuter, 1983), cAMP elevation leads to the activation of protein Kinase A phosphorylating L-type Ca^{2+} channels which in consequence is enhanced. However, alternative pathways for an indirect muscarinic inhibition of L-type Ca^{2+} channel have been postulated. Thus, cGMP dependent effects have been described (Hartzell and Fischmeister, 1986) such as cGMP inhibiting L-type Ca^{2+} channels that previously had been increased by cAMP. A role for a c-GMP stimulated phosphodiesterase PDE2 has been suggested (Simmons and Hartzell, 1988). In additional experiments, it was shown that L-type Ca^{2+} channels can be inhibited by cGMP in a manner independent from changes in cAMP levels (Thakkar et al., 1988). In contrast, others could not find an effect of cGMP on L-type Ca^{2+} channels without prior stimulation by isoprenaline or cAMP (guinea-pig ventricle [Levi et al., 1989]). Besides these findings, cGMP dependent activation of cAMP-dependent phosphodiesterase (Mery et al., 1993) or activation of cGMP- dependent protein kinase (PKG) finally leading to reduction in L-type Ca^{2+} channels (Mery et al., 1991) have been reported. Recently, it has been shown that in guinea pig ventricular cells carbachol inhibits L-type Ca^{2+} channels via cGMP/PKG-dependent activation of an okadaic acid-sensitive protein phosphatase. This effect of carbachol could be counteracted by elevated intracellular levels of cAMP (Sakai et al., 1999). Similarly Shirayama and Pappano (1996) described an inhibition of L-type Ca^{2+} channels by a cGMP-dependent protein kinase in guinea pig ventricular myocytes. While some of the first studies mentioned above were performed

in frog heart, more recent studies were carried out using mammalian and human cardiomyocytes. Thus, it has been shown that L-type Ca^{2+} channel in human atrium is controlled via phosphodiesterase 2 (PDE2) and that inhibition of PDE2 caused increases in L-type Ca^{2+} channels, but only in the presence of GTP (Rivet-Bastide et al., 1997). This effect was seen only in human atrial or ventricular myocytes. In addition, the effects of muscarinic stimulation on L-type Ca^{2+} channel and $I_{\text{k,ACh}}$ was inhibited by methylene blue (a blocker of guanylylcyclase), in rat ventricular and atrial cells (Abi-Gerges et al., 1997). However, it should be kept in mind that methylene blue can act as an antimuscarinic agent (Abi-Gerges et al., 1997).

It has been shown that ACh can reduce isoprenaline-induced phosphorylation of intracellular proteins (Iwasa and Hosey, 1983) such as troponin (England, 1976) or phospholamban (Lindemann and Watanabe, 1985). Reduced phosphorylation, however, could also mean increased activity of phosphatases. Indeed, Ahmad et al. (1989) could demonstrate an increase in activity of type I phosphatase in guinea-pig ventricle following stimulation with ACh. Consistent with that finding, others observed a diminution of the indirect ACh-induced negative inotropic effect by NaF, a substance which can inhibit protein phosphatases (Neumann et al., 1995). Another substance known to inhibit phosphatases, okadaic acid, was shown to prevent the reduction in protein phosphorylation induced by ACh (Gupta et al., 1994). A possible functional relevance of these findings was demonstrated by Herzig et al. (1995), who showed that ACh-induced inhibition of L-type Ca^{2+} channels involves stimulation of protein phosphatase. In the human ventricles, muscarinic stimulation does not seem to directly inhibit L-type Ca^{2+} channels since a direct negative inotropic effect has not been

observed (Bohm et al., 1989). However, indirect antagonism on β -adrenergic stimulation of contractile force has been found in the human ventricle (Giessler et al., 1999).

The typical effects of ACh on supraventricular tissue resulting in membrane hyperpolarization and slowing of pacemaker activity have been classically attributed to the activation of $I_{k,ACh}$ as the main underlying mechanism (Trautwein, 1981). However, it was shown that low concentrations of ACh shift the current activation curve for the pacemaker current I_f to more negative potentials, thus inhibiting I_f in sinus nodal cells. This is the opposite of the action of catecholamines (acting via cAMP). It is assumed that muscarinic receptor stimulation leads to I_f inhibition via a pertussis-toxin sensitive G-protein, resulting in inhibition of adenylyl cyclase and reduced cAMP production. This alters the I_f availability, since this channel is cAMP sensitive. DiFrancesco and Tromba (1988) investigated the concentration-dependent effects of ACh on both currents, revealing that at low concentrations (0.01 μ M) ACh inhibits I_f but has no effect on $I_{k,ACh}$, which is functionally reflected by a slowing of pacemaker rate without hyperpolarization, while at concentrations of $> 0.1 \mu$ M ACh inhibits I_f and activates $I_{k,ACh}$, leading to both slowing of pacemaker rate and hyperpolarization. The half-inactivating concentration (IC_{50}) (for I_f) of ACh was 0.013 μ M and IC_{50} of $I_{k,ACh}$ was 0.260 μ M in these experiments. This indicates that ACh-mediated inhibition of I_f contributes in an important manner to the slowing effect of low ACh concentrations on sinus nodal rate (DiFrancesco, 1990). Besides cAMP dependent G_s protein mediated regulation of I_f (with a slow time course as can be expected for an effect involving a second messenger) a direct effect of muscarinic cholinergic activated G_o proteins (cAMP independent and with a faster time course) on I_f has also been supposed (Yatani

et al., 1990 a and b). Thus, the effect of ACh on *I_f* may comprise two components: a slow but large cAMP dependent component and a fast but small cAMP independent component caused by coupling of muscarinic receptors with the channel via Go proteins. This action seems to be mediated by the α subunit (Yatani et al., 1990 a and b). In human heart there is also direct inhibition of *I_f* by muscarinic stimulation, as has been shown in isolated human atria myocytes using carbachol (Hoppe et al., 1998). In the human ventricles this current is also present. However, in contrast to human atrium there is no direct effect of muscarinic stimulation on human ventricular *I_f* (Hoppe and Beuckelmann, 1998).

ACh hyperpolarizes the vascular smooth muscle cell membrane in an endothelium-dependent manner by the release of mediators such as NO, endothelium derived hyperpolarizing factor, and prostacyclin. ACh-induced hyperpolarization is not generated in high extracellular K^+ concentration ($[K^+]_o$) solution, indicating that vascular smooth muscle hyperpolarization by endothelial factors is mediated by K^+ channel activation, which in turn closes voltage-dependent Ca^{2+} channels, reduces cytosolic Ca^{2+} concentration and induces vasodilatation. NO and endothelium-derived hyperpolarizing factor (EDHF)-mediated responses can also involve the Na^+ , K^+ -ATPase that is found in the plasma membrane of vascular smooth muscle cells (Busse et al., 2002) and is thought to be critically involved in the maintenance of cellular ionic homeostasis needed to regulate membrane potential and vascular smooth muscle tone (Marin and Redondo, 1999). Membrane hyperpolarization and increased cytosolic Ca^{2+} concentration has been reported in endothelial cells stimulated by ACh (Busse et al., 1988). However, BK_{Ca} blockers have only a marginal effect on cytosolic Ca^{2+} in endothelial cells, indicating

that these blockers most likely act on K^+ channels of the vascular smooth muscle cells (Yamanaka et al., 1998). To attribute the effects of BK_{Ca} channel blocker on ACh responses to actions on aortic vascular smooth muscle, Callera et al. (2004) showed that Iberitoxin (IbTX) (big conductance K_{Ca} channel blocker) inhibits SNP vasodilatation only in endothelium denuded aortic rings from 2-kidney (with no clip) rats without affecting the response on vessels from 2K-1C rats. The small conductance K_{Ca} channels are involved in the endothelium-dependent relaxation. The association of apamin (small conductance K_{Ca} channel blocker) and IbTX produced an inhibition of ACh relaxation, compared with the effect of IbTX alone, only in aortic rings from 2K-1C rats. The higher total amount of intracellular Ca^{2+} after K_{Ca} blockade contributes to the activation of small conductance K_{Ca} channels in 2K-1C aortas. Therefore, apamin-sensitive K_{Ca} channels contribute to ACh-induced relaxation, instead of being responsible for the impaired ACh response, in aortas from 2K-1C (Callera et al., 2004).

1.7. RATIONALES OF THE STUDY

1. **In a previous study, we demonstrated that nicotine, an exogenous substance, stimulated or inhibited, depending on the concentrations, the whole-cell K^+ channels in rat tail artery SMCs (Tang et al., 1999).** Since the molecular nature of K^+ channels in the previous study was not clear, the altered whole-cell K^+ currents in the presence of nicotine could be conducted by K_v channels, K_{Ca} channels or K_{ATP} channels. It was consequently questioned whether nicotine could interact with K_{ATP} channels, and more specifically with Kir6.1 subunits.
2. **Wang and colleagues (2000a) demonstrated that nicotine blocked multiple types of K^+ currents (including A-type currents, delayed rectifier current and inward rectifier current) with preferential inhibition of transient outward currents ($I_{to}/K_v4.3$) and those results indicate that nicotine is a non-specific blocker of K^+ channels with certain selectivity toward A-type currents.** The effects of nicotine were independent of nicotine receptor stimulation or catecholamine release and the effects were not reversed or prevented by mecamlamine (100 μ M, nicotine receptor antagonist), atropine (1 μ M, muscarinic receptor antagonists), or propranolol (2 μ M, β -adrenoceptor blocker). Thus the inhibitory effects are likely the consequence of direct interactions between nicotine molecules and the channel protein. Accumulating evidence has shown that nicotine can also exert its effects without involvement of nAChRs and catecholamine release. Studies under conditions devoid of nAChR stimulation demonstrated the ability of nicotine to alter action potential

(AP) characteristics in guinea pigs (Pappano and Rembush, 1970), rabbits (Carryl et al., 1992), and dogs (Greenspan et al., 1969) in different tissues such as sinus nodes (Satoh, 1997), atrium, ventricle, and Purkinje fibers. The most noticeable changes were decreases in resting potential and prolongation of later AP phases. It is therefore quite conceivable that nicotine might be able to interact directly with ion channels.

3. **Mayhan and Sharpe (2002) reported that acute and chronic exposure to nicotine has profound affects on vasodilatation induced by activation of K_{ATP} channels.** Chronic exposure to nicotine did not alter vasodilatation induced by nitroglycerin but impaired vasodilatation induced by aprikalim and cromakalim (K_{ATP} channel openers). In nicotine-treated hamsters aprikalim and cromakalim failed to dilate the arterioles. In addition, Mayhan and Sharpe (2002) reported that acute treatment of the cheek pouch microcirculation with SOD attenuated the effects of nicotine on aprikalim and cromakalim induced vasodilatation. Since K_{ATP} in vascular tissues is mainly formed by Kir6.1 subunits, these data suggest that nicotine modulates Kir6.1 channels.
4. **ACh is an important endogenous neurotransmitter and vascular relaxant.** In our preliminary study, we applied ACh to HEK-293 cells that expressed heterologous Kir6.1 channels. Surprisingly, ACh stimulated Kir6.1 channels.

1.8. WORKING HYPOTHESES

1. Nicotine and ACh modulate the function of the stably expressed K_{ATP} channel subunit (Kir6.1) and such modulation is independent of acetylcholine receptors.
2. Nicotine modulation likely involves mediatory roles of free radicals, particularly superoxide anion and such mechanism is not provoked in the ACh modulation of the channel.

1.9. OBJECTIVES OF THE STUDY

Based on the aforementioned rationales and hypotheses, the following objectives were undertaken.

1. To establish a cell line of heterologously expressed Kir6.1 channels in HEK-293 cells and to characterize and confirm the expressed Kir6.1 channels.
2. To study the modulation of cloned Kir6.1 channels by nicotine and the underlying mechanisms.
3. To study the effects of O_2^- on the expressed Kir6.1 channels.
4. To study the modulation of Kir6.1 channels by ACh and the underlying mechanisms.

2. GENERAL METHODOLOGY

2.1. CELL PREPARATION

2.1.1. Stable transfection of HEK-293 cells with Kir6.1 gene

HEK-293 cells (American Type Culture Collection, Rockville, MD) were cultured in 35 mm Petri dishes as described previously (Cao et al., 2002). The pCR2.1-rvKir6.1 cDNA clone (GenBank # AB043637) was cleaved with appropriate restriction endonucleases to produce the cloned rvKir6.1 subunit gene insert with proper restriction enzyme cleavage sites at both ends. It was then subcloned into a modified mammalian expression HA3 vector (King et al., 2000). The construct was linearized with Eam 1105I restriction endonuclease (MBI Fermentas). Linearized construct was mixed with a FuGENE™ 6 transfection reagent (Roche) in a ratio of 1 (µg): 3 (µl) in 100 µl of fetal bovine serum (FBS) -free RPMI-1640 medium. After incubating for 45 min at room temperature, the mixture was added to HEK-293 cells in 2 ml FBS-free RPMI medium (cell density: 8×10^4 /35 mm dish). Geneticine (GIBCO) selection was performed at concentration of $500 \mu\text{g ml}^{-1}$. Non-transfected HEK-293 cells were included as a negative control for antibiotic selection. After 4 weeks of antibiotic selection culturing, surviving transfected cells were picked individually into 24-well culture plates for proliferation. When the cells became >90% confluent in 90 mm culture dishes, they were harvested and frozen in liquid nitrogen for electrophysiological study. Stable transfection took around 6 days to complete:

A. Day 1: We washed the stock plate of cells with 5 ml physiological saline solution (PBS) and we add trypsin to detach the cells. Then we resuspended the trypsinized cells in 10 ml standard media to make an even suspension. In 10 ml PBS we made cell suspensions with 10 μ l aliquot of the cells. We added 2 ml of fresh standard media into three 6.0 cm Petri cell culture dishes (one for transfection, one for vector only transfection and one for the negative control). We pipetted the volume of cells into each plate containing the media (usually 10^5 – 10^6 cells for each dish) and we rocked the dishes back and forth to distribute the cells evenly over the surface. We incubated the dishes overnight in the incubator at 37°C and 5% CO₂.

B. Day 2: We performed the transfection in the second day by preparing the following solution in sterile Eppendorf tubes for each Petri dish: Solution A: 300 μ l non-FBS MEM media plus 2.0 μ g DNA (must be of transfection quality i.e, made by Qiagen Prep kits or equivalent and properly linearized). Solution B: 300 μ l non-FBS media plus 6 μ l Fugene6 (Roche) transfection reagent. We combined solutions A and B, mixed them gently, and incubated at room temp. for 30 min. We added 2.4 ml non-FBS MEM to the cells in the Petri dishes and then gently we added the above combined solutions above down to the side of the plates. For the negative control dish, we just added 3 ml of non-FBS MEM and incubated as well in the incubator at 37°C and 5% CO₂ for 6 hours. After 6 hours of incubation we added 3 ml of MEM + 20% FBS + pen/strep medium without removing the transfection media and also added the same amount of the media to the mock transfected cells and negative control dishes. We incubated the cells in the incubator at 37°C and 5% CO₂ overnight.

C. Day 3: We changed the media and replaced it with normal MEM +10% FBS + pen/strep in both transfected dish and control dish.

D. Day 4 to 6-splitting cells and the antibiotic selection: When the transfection and the control dish are confluent, we washed the cells with PBS and trypsinized them as usual. We resuspended the cells in 10 ml of selective media for each plate. The selective media is the media the cells normally grow in, with the addition of high concentrations of the selection drug. We split these cells onto 10-10 cm dishes by adding 9 ml of the selective media and 1 ml cell suspension. We rocked the dishes to distribute the cells evenly. We allowed the cells to grow at 37°C and 5% CO₂ for 7-14 days (typically 10 days) while replacing the selective media every 3-4 days, until distinct colonies of drug resistant cells can be seen in the transfected dish. Usually the cells in the negative control dish had all died at this point in time, allowing us to discard these dish (es).

E. Picking the colonies: We removed the media from all the plates of cells. We washed each plate with 5 ml PBS and then aspirated off all the PBS. We examined the colonies and selected isolated ones with a marker, choosing no more than 3 colonies from each plate. We used sterile forceps to pick up a sterile 3mm filter paper circle and dipped it into the freshly made trypsin. We placed this circle directly on top of a cell colony that we had previously circled with a marker. Trypsin dislodged the cells from the plates. We prepared 24 well dishes with media. We transferred the paper disk, with associated cells, into one of the prepared wells in the 24 well dishes. We rocked the 24 well dishes to distribute the cells evenly. We allowed the cells to grow in the incubator

at 37°C and 5% CO₂ until the cells were confluent.

F. Scaling up the cell colonies for analysis and stocks: When the wells become confluent, we split them. Each cell colony was split into 2 samples. A larger cell sample on a 6 well plate was used for lysis and analysis. A smaller cell sample was kept on a stock plate so that they would be readily available after Western analysis/patch clamp study. Therefore, care was taken not to contaminate those stocks since they were the only sample of that particular clonal cell line.

G. Storage of cell lines: When the cells were confluent, they were harvested and frozen in liquid nitrogen.

2.2. ELECTROPHYSIOLOGICAL RECORDING OF THE WHOLE-CELL K_{ATP} CHANNEL CURRENTS (PATCH-CLAMP TECHNIQUE)

2.2.1. Solutions

The pipette (intracellular) solution contained (mM): 132 KCl, 1.2 MgCl₂, 1 CaCl₂, 10 ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'- tetraacetic acid (EGTA), and 5 Hepes. Na₂ATP 0.3 or 5 mM was added to the pipette solution immediately before the experiments. The bath (extracellular) solution contained (mM): 40 KCl, 100 NaCl, 2.6 CaCl₂, 1.2 MgCl₂, and 5 Hepes. Before use, all solutions were filtered through a filter with a pore size of 0.22 µm. The pH of both bath and pipette

solutions was adjusted to 7.4 with HCl for the bath, and NaOH for the pipette solutions, respectively.

2.2.2. Whole-cell recording

2.2.2.1. Recording and off-line analysis of current records

The current traces were digitized using a personal computer system. pClamp, version 7.0 (software package purchased from Axon Instruments, Inc.) was used to record the currents using an Axopatch-200B patch clamp amplifier (Axon Instruments, Union City, CA, USA). The output signals were filtered at 1 KHz with an 8-pole filter, and stored on the hard disk of a computer for off-line analysis. Depolarization pulses were generated by a computer through a digital-to-analog converter (Fig. 2.1) (Wang and Wu, 1997; Wang et al., 2000b).

2.2.2.2. Procedures of current recording

Pipette: The pipette for recording in HEK-293 cells was made of borosilicate thin wall glass microhematocrit capillary tubes (OD = 1.2 mm, ID = 0.9 mm, Fisher Scientific, ON) and had a resistance of 2-4 M Ω (Wang et al., 2001; Wang et al., 1993). These pipettes were pulled by a two-stage electrode puller (Sutter Instruments CO, USA, Model P-87).

Seal penetration: The standard gigaohm seal, whole-cell version of the patch-clamp technique was used to measure whole-cell K_{ATP} currents (Sun et al., 2004). A

Petri dish with attached cells was mounted on the stage of an inverted phase contrast microscope (Olympus, Carsen Group Inc., ON, Canada). The output signals were filtered at 1 KHz with an 8-pole filter, and stored on the hard disk of a computer for off-line analysis. The pipette, which had a small positive internal pressure in order to keep the tip clean, was pushed onto the cell surface by using a three dimensional micromanipulator (MP-285, Sutter Instrument Company). A tight seal (gigaseal) between the membrane and the tip of the pipette (10 to 50 gigaohm) was obtained by the application of suction through the pipette (Hanna and Wang 2002). Further suction disrupted the membrane under the tip of the pipette, and then the pipette solution dialysed the cell. During the process of forming a gigaohm seal between the tip of the pipette and the membrane patch, some cells were sucked to such a degree that a gigaohm seal could not be achieved. These cells could not be used. The experiments were carried out at room temperature (20-22 °C) in order to ensure a longer survival time of patched cells and a better time resolution of the membrane currents. The membrane currents of HEK-293 cells were amplified by an Axopatch-200B (Axon Instrument, Inc., USA) patch clamp amplifier.

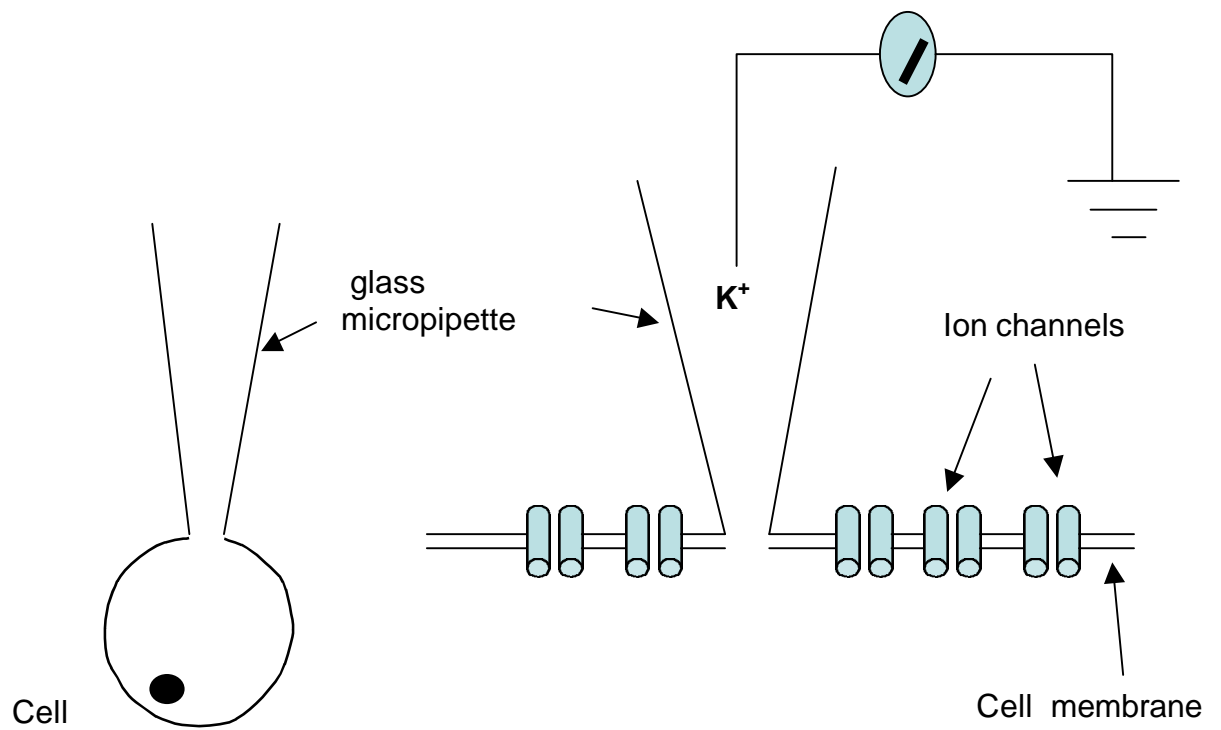


Figure 2.1. Schematic diagram of whole cell patch-clamp recording.

Run-down: In the present study, much effort was devoted to exclude the interference of “run-down” of K_{ATP} channel currents. Run down is a gradual, variable, and probably multifactorial, reduction of channel activity, often associated with decreased open probability and increased $K_{1/2,ATP}$ (ATP causing half-maximal inhibition) (Kamouchi and Kitamura, 1994). If the current magnitude did not change from the third to the fifth minute after the rupture of the cell membrane, the rate of “run-down” of K_{ATP} current was usually negligible for 20 min or longer. Cells that had stable K_{ATP} currents from the third to fifth minute after penetration of the membrane were used. Drugs were tested once the whole-cell currents had reached a stable level (i.e. >10 minutes after obtaining the whole-cell configuration). If the current decayed too quickly, it was difficult to distinguish “run-down” of the current from the inhibitory or excitatory effect of the agents. Cells that had a fast decline of K_{ATP} currents within this period were discarded. A series of voltage steps from -150 to $+80$ mV were applied to determine the current-voltage relationship. Current density was calculated by dividing the current amplitude over the cell capacitance. At the end of the first five minutes the I-V relationship was plotted as control (Wang and Wu, 1997). Different drugs were then added directly to the bath solution and the change in K_{ATP} current was monitored continuously. In some cells the bath solution was changed to wash out the tested agent in order to determine whether the effects of these agents were reversible. This procedure further excluded the influence of “run-down” of K^+ channel currents. No allowance, hence, was made for the spontaneous decline of K_{ATP} current in measuring the effect of various agents on K_{ATP} channel currents.

2.3. MATERIALS AND WASHOUT PROCEDURE

Stock solutions of nicotine, ACh, cytosine and 1, 1-dimethyl-4-phenylpiperazinium iodide (DMPP) were prepared in distilled water and protected from light. The cells were perfused with the freshly made bath solutions containing the desired concentrations of nicotine or ACh. In some experiments, the required concentration of the drugs was obtained in a 3 ml bath dish by adding a specific amount of the stock solution to the dish. Care was taken that the needed amount of the drugs added ranged between 10-30 μ l. In some other experiments drugs were added in the perfusion system. Only one experiment was conducted per dish. The washout protocol was executed to examine the reversibility of the effects of the agents on K_{ATP} channel currents (Wang et al., 1991; Wu et al., 1992). Satisfactory washout of the agent from the bath solution was obtained with a perfusion rate of approximately 1 ml/10 sec.

2.4. QUANTIFICATION OF SUPEROXIDE ANION PRODUCTION BY LUCIGENIN CHEMILUMINESCENCE

Generation of superoxide radicals was measured using a LUCY-1 luminometer (Kober et al., 2003). Briefly, HEK-293 cells (2.5×10^6 cells/250 μ l) were equilibrated in Krebs buffer (pH 7.4, 37°C) for 30 min in micro-centrifuge tubes in the water bath and then 250 μ M lucigenin was added to each tube for 5 min. Thereafter, nicotine at different concentrations was added to each tube. Superoxide production in the cells was detected after 10 min incubation with nicotine by determining the average luminescence

counts for ten measurements with an integration time of 4 s for each measurement. Control experiments were included in which cells were incubated with lucigenin for the same period of time but no nicotine was added. The background luminescence was determined by mixing lucigenin and nicotine in the buffer without cells. This background luminescence had been compensated for in all luminescence count results. Tempol (4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl), a novel stable nitroxide (antioxidant) (100 μ M), was added to the tubes in some experiments before the addition of nicotine to quench superoxide anion produced (Fig. 2.2).

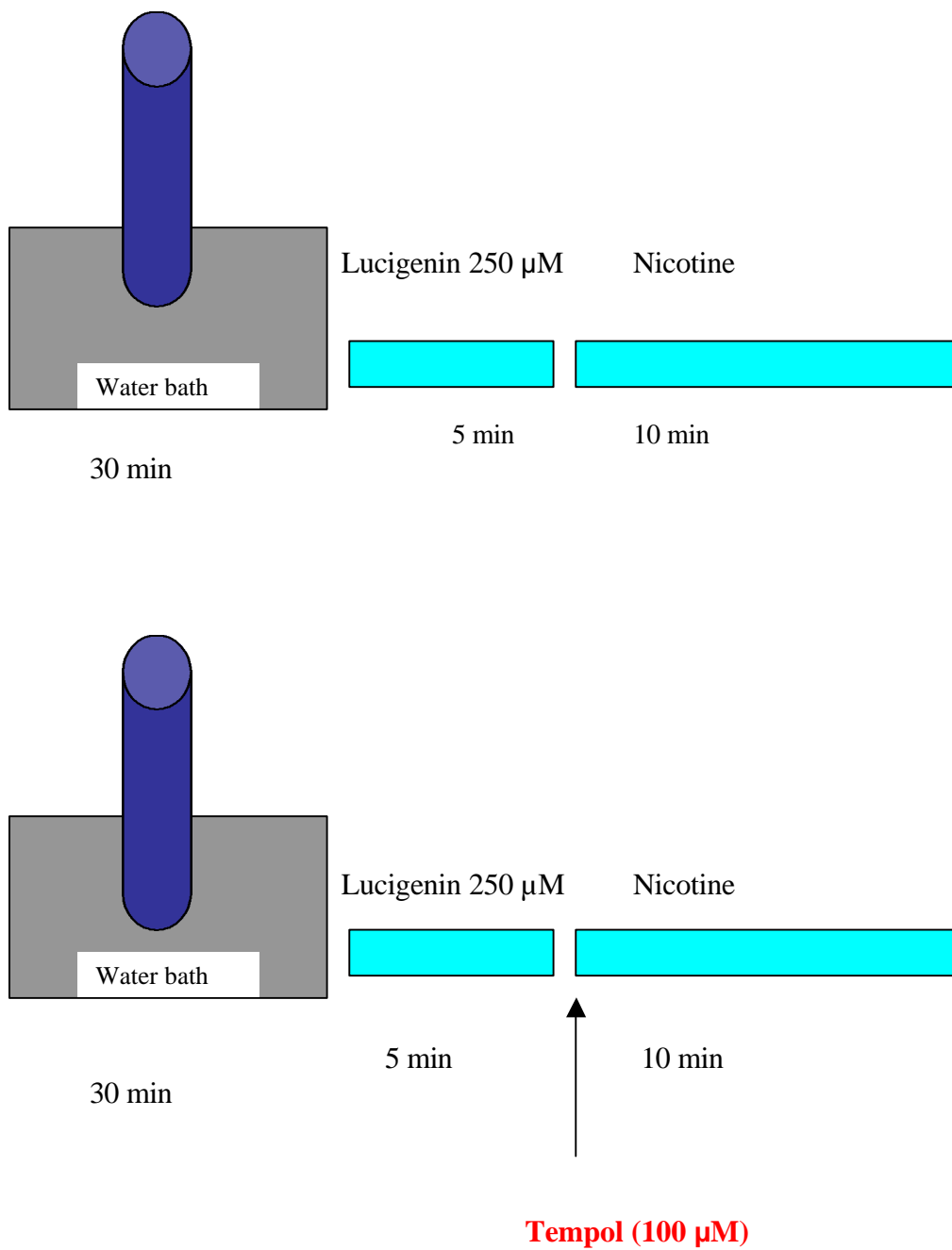


Figure 2.2. Schematic diagram of the measurement of superoxide anion ($O_2^{\cdot -}$) production.

2.5. STATISTICAL ANALYSIS

Unless original traces or single experiments are shown, values are expressed as means \pm SE. The data from studies on the Kir6.1 currents are expressed as either absolute values or percentage of the pre-drug control condition. Concentration-response curves were fitted to a Hill equation using a computerized curve fitting software (Microcal Origin, version 5.0, Microcal Software, Northampton, MA) to obtain the concentration at which half-maximal inhibition occurred, IC₅₀.

The Hill equation is a fundamental expression in chemical kinetics relating velocity of response to concentration. It is known that the Hill equation is parameter indefinable which means that “perfect data” that is a complete Hill curve, uniquely determine the three parameters V_{\max} , K_M , and V (Heidel and Maloney, 1999). The Hill equation is frequently used in pharmacology to describe the response of an organism or tissue as a function of drug concentrations:

$$V = \frac{V_{\max} [S]^n}{K_M + [S]^n}$$

Where V is the pharmacologic response at drug concentration $[S]^n$, V_{\max} is the maximum possible pharmacologic response, K_M is the concentration of drug at which $V = 1/2 V_{\max}$ and is also a measure of the affinity of the receptor for the drug. The parameter n , the Hill coefficient, can be viewed mechanistically as the number of molecules that bind to a receptor. More frequently it is regarded functionally as a factor

determining the response curve (Heidel and Maloney, 1999; Shen, 2000). The paired Student's *t*-test or group *t*-test was used for comparison between mean values of the control and those obtained after drug administration. In the case of multiple amplitude comparisons, analysis of variance in conjunction with the Newman-Keul's multiple range tests was applied. A significant difference level was set at $p < 0.05$.

3. INTERACTION OF NICOTINE WITH HETEROLOGOUSLY EXPRESSED KIR6.1 CHANNELS IN HEK-293 CELLS

3.1. INTRODUCTION

Consumption of tobacco adversely affects cardiovascular system in humans. Increased risk of atherosclerotic vascular disease, hypertension, myocardial infarction, unstable angina, sudden cardiac death, and stroke are among many pathological situations associated with tobacco use. The most significant substance that affects cellular function in cigarette smoking and smokeless tobacco is nicotine. Yet the cellular effects of nicotine and the underlying mechanisms are still to be clarified. Nicotine is absorbed rapidly through oral mucosa in smokeless tobacco users. Absorbed nicotine may release catecholamine from sympathetic nerve endings. The subsequent activation of α -adrenoceptors in vascular smooth muscle cells (SMCs) contracts vascular tissues and elevates blood pressure (Toda et al., 1995). Nicotine may also act directly on vascular SMCs to induce vascular relaxation or contraction (Wang and Wang, 2000). These effects of nicotine would contribute to altered cardiovascular function with tobacco consumption.

It has been shown that nicotine has toxic effects on the endothelium (Lin et al., 1992; Lakier, 1992). Acute and chronic treatments with nicotine specifically impair nitric oxide synthase-dependent vasodilatation and the release of nitric oxide (NO) by microvessels (Mayhan and Sharpe, 1999). The mechanism by which nicotine alters the NO-dependent response of blood vessels appears to be related to the production of oxygen radicals, which presumably inactivate NO (Raij et al., 2001). It is thus reasoned that nicotine may affect cellular functions by altering the oxidative stress level.

In addition to synthesis and release of NO, relaxation of blood vessels may also occur via activation of K_{ATP} channels. K_{ATP} channels are closed by increases in intracellular ATP. These channels are therapeutic targets for several diseases, including angina, hypertension, and diabetes. Stimulation of K_{ATP} channels produces vasorelaxation and myocardial protection against ischemia, whereas its inhibition facilitates insulin secretion. K_{ATP} channels are octameric complexes of 2 distinct subunits, a sulfonylurea receptor (SUR) and pore-forming Kir6.x subunits (Cao et al., 2002). SUR confers high-affinity block by sulfonylureas and stimulation by K^+ channel openers (KCO) and MgADP. ATP inhibits channel activity through an interaction with the Kir6.x subunit. The expressed Kir6.1 channel is only slightly stimulated by diazoxide (K_{ATP} channel opener) but not inhibited by glibenclamide (K_{ATP} channel blocker) (Ammala et al., 1996a). There is also no study reporting the effects of tobacco consumption or nicotine on the activation of K_{ATP} channels.

3.1.1. Objectives of this study

In a previous study, we demonstrated that nicotine stimulated or inhibited, depending on the concentrations, the whole-cell K^+ channels in rat tail artery SMCs (Tang et al., 1999). Coincidentally, nicotine also evoked concentration-dependent contraction or relaxation of the isolated arterial tissues (Wang and Wang, 2000). Since the molecular nature of K^+ channels in the previous study was not clear, the altered whole-cell K^+ currents in the presence of nicotine could be conducted by voltage-gated K^+ channels, calcium-sensitive K^+ channels or K_{ATP} channels. It was consequently questioned whether nicotine could interact with K_{ATP} channels, and more specifically

with Kir6.1 subunits. Thus, the first goal of the present study was to examine the effect of nicotine on the cloned Kir6.1 subunit, which was heterologously expressed in HEK-293 cells. The second goal was to examine the mechanism(s) by which nicotine modulated the function of K_{ATP} channels.

3.2. MATERIALS AND METHODS

3.2.1. Stable transfection of HEK-293 cells with Kir6.1 gene

Details are described in section 2.1.

3.2.2. Electrophysiological recording of the whole-cell K_{ATP} channel currents

Details are described in sections 2.2. and 2.3.

3.2.3. Quantification of superoxide anion production

Details were described in section 2.4.

3.2.4. Data analysis

The chemicals we used are listed in page xx under the (List of chemicals). The Superoxide production was expressed as count per minute (cpm)/10⁶ cells. Details of the statistical analyses are described in section 2.5.

3.3. RESULTS

3.3.1. Properties of the expressed Kir6.1 channel currents

HEK-293 cells possessed an endogenous background K^+ channel with current density of -3.2 ± 1.4 pA/pF at -150 mV ($n = 9$). The amplitude of this current did not change significantly during 15 min of dialysis with an intracellular solution containing 0.3 mM ATP. In cells transfected with Kir6.1 cDNA, the whole-cell current had a significantly greater density (-23.9 ± 1.6 pA/pF at -150 mV, $n = 6$) and this current did not rundown after 15 minutes of dialysis with 0.3 mM intracellular ATP (-24.1 ± 1.8 pA/pF) (Fig. 3.1A). Dialysis of HEK-293 cells expressing Kir6.1 channels with 5 mM ATP in the pipette solution caused a decrease in current density although not significantly (-22.43 ± 4.7 vs. -16.42 ± 2.3 pA/pF, $n = 8-5$ for each group, $p > 0.05$) (Fig. 3.1B). Inward rectifier K^+ (Kir) channels are very sensitive to inhibition by extracellular Ba^{2+} (Nelson and Quayle, 1995). In our experimental conditions Ba^{2+} at 0.5 mM inhibited Kir6.1 currents by 52.5% (Fig.3.2). PNU-37883A, a morpholinoguanidine, is a novel K_{ATP} channel inhibitor. This compound appeared to have selectivity for the Kir6.1-based vascular K_{ATP} channel (Kovalev et al., 2004). Application of 30 μ M PNU-37883A to the transfected HEK-293 cells markedly inhibited Kir6.1 currents (-37.4 ± 2.8 vs. -25.85 ± 2.9 pA/pF at -150 mV, $n = 6$, $p < 0.05$). The effects of PNU-37883A were reversible (Fig. 3.3).

The identity of heterologously expressed Kir6.1 channel was further confirmed using a specific anti-Kir6.1 antibody (Kir6.1 Ab). GST-tagged Kir6.1C fusion protein was used as antigen to raise antisera (Sun et al., 2004). The antisera were pre-absorbed

first with GST protein before being used in the following Western blot experiments. A single 41 kDa band of Kir6.1 protein was detected in HEK-293 line, which was permanently transfected with rvKir6.1 coding cDNA (lane 2 in Fig. 3.4A and B). Interestingly, a band with the same molecular mass was also detected in non-transfected native HEK-293 (lane 1 in Fig. 3.4A and B). Quantitative analysis revealed that the expression levels of Kir6.1 proteins in the transfected HEK-293 cells were 5.3-fold higher than that in non-transfected HEK-293, when normalized with β -actin expression level ($P<0.01$) (Fig. 3.4B). Furthermore, the antibody specifically recognized a single 38 kDa bacterial Kir6.1C–GST fusion protein (Fig. 3.4A, lane 3) and did not cross-react with a Kir6.2C–GST fusion protein (Fig. 3.4A, lane 4). After the antibody was pre-absorbed with Kir6.1 bacterial fusion protein prior to Western blot experiment, positive bands in HEK-293 cells disappeared (Sun et al., 2004). When anti-GST antibody was used as primary antibody instead of anti-Kir6.1 antibody, only Kir6.1–GST fusion protein was detected (Fig. 3.5A). No reaction was observed between anti-GST antibody with any tested cells or tissues (Fig. 3.5A and B).

We used Kir6.1 Ab to confirm the identity of the expressed Kir6.1 channels. Because the Ab and the serum were included in the pipette solution we were not able to record the control current as a separate value as we did in all other experiments. Therefore, the whole cell recording started after the formation of the whole-cell recording configuration (i.e. after membrane break). The control was considered to be the first 50 seconds of the recording. After that the effect of the Ab starts to appear due to the mixture of the Ab (or the serum) included in the pipette and the intracellular cytoplasm. Two minutes after the formation of the whole-cell recording configuration,

the cells were repetitively hyperpolarized to -150 mV from a holding potential of -20 mV with 800 ms pulses applied at 1 kHz. Kir6.1 currents were stable for 15 min of recordings in the presence of 1:500 control sera in the pipette solution (Fig. 3.6A). Control serum was taken from the same rabbit before immunization with Kir6.1 antigenic proteins (Sun et al., 2004). With Kir6.1 Ab (1:500) in the pipette solution, Kir6.1 currents were inhibited within 10 min after the establishment of the whole-cell configuration by $21.3 \pm 4.6\%$ (-927.7 ± 25.9 to -729.7 ± 42.9 pA) (Fig. 3.6B). The inhibition of Kir6.1 currents became stable 10-15 min thereafter.

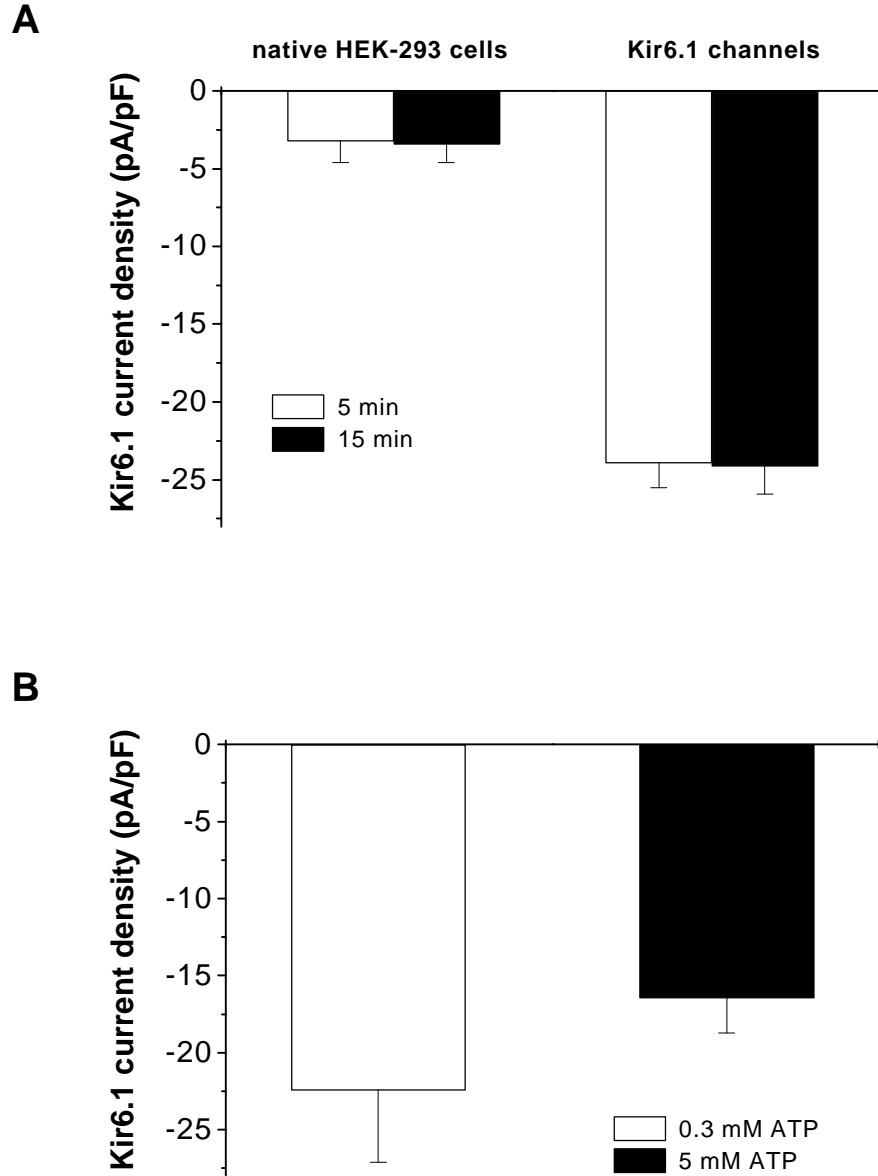


Figure 3.1. Properties of the endogenous and the expressed Kir6.1 channel currents in HEK-293 cells (test potential, -150 mV; holding potential, -20 mV). **A.** K^+ channel current in native non-transfected and Kir6.1 cDNA transfected HEK-293 cells after 5 and 10 min of recording with 0.3 mM ATP in the pipette solution (n = 9-6). **B.** Kir6.1 currents with 0.3 and 5 mM ATP in the pipette solution (n = 8-5).

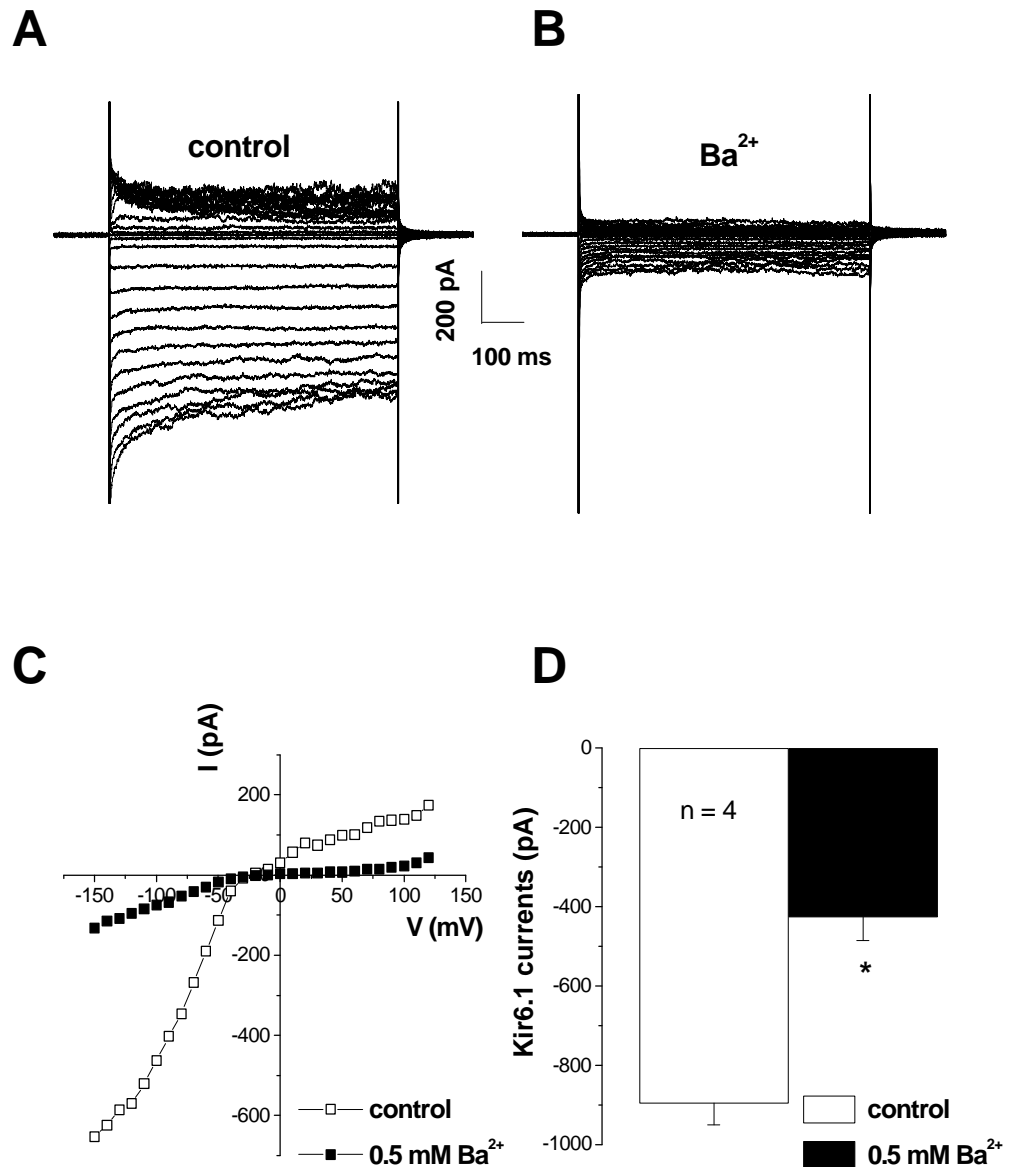


Figure 3.2. Effect of Ba^{2+} on Kir6.1 channels stably expressed in HEK-293 cells. Original recordings of Kir6.1 currents before (A) and after (B) the application of 0.5 mM Ba^{2+} to the bath solution. C. I-V relationship of Kir6.1 channels before and after application of 0.5 mM Ba^{2+} . D. Summary of the effects of Ba^{2+} on Kir6.1 channels at -150 mV. * $p < 0.05$.

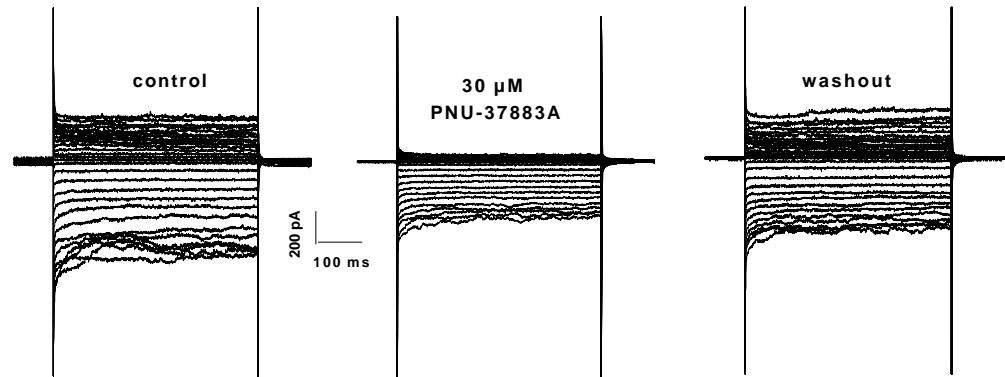
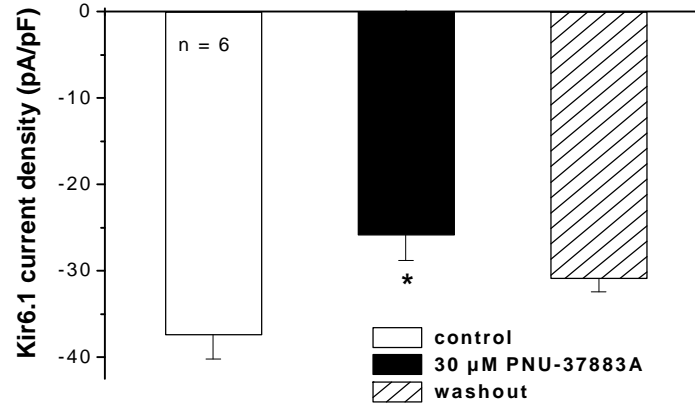
A**B**

Figure 3.3. Effect of K_{ATP} channel blocker PNU-37883A on Kir6.1 channels stably expressed in HEK-293 cells. **A.** Original recordings of Kir6.1 currents before, during, and after the application of 30 μ M PNU-37883A to the bath solution. **B.** Summary of the effects of PNU-37883A on Kir6.1 channels at -150 mV. * $p < 0.05$.

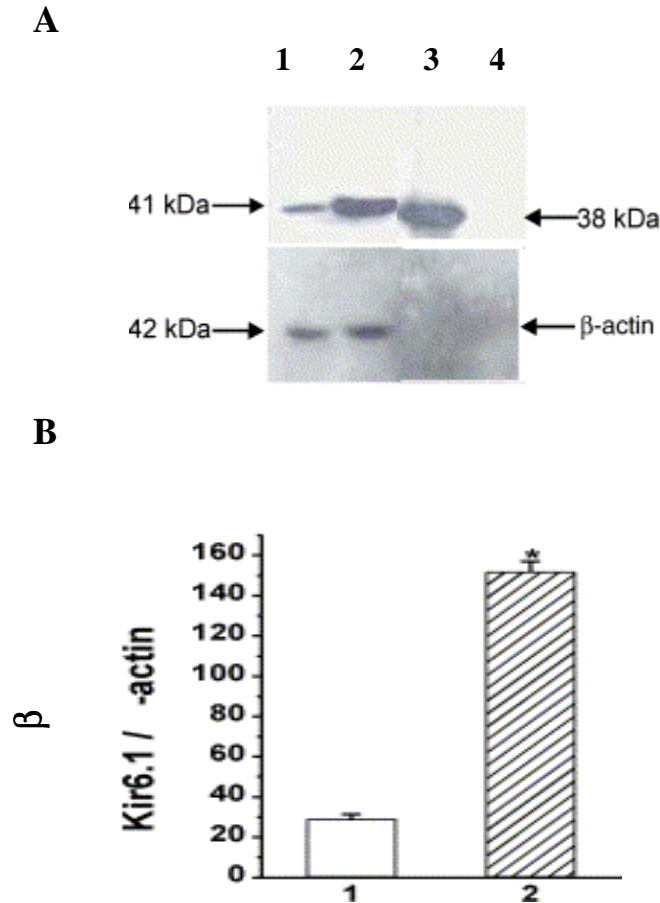
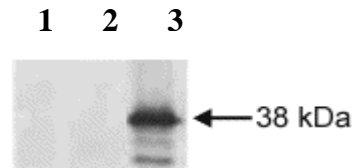


Figure 3.4. Detection of Kir6.1 protein in HEK-293. **A.** Representative Western blots with anti-Kir6.1 antibodies. Lane 1, non-transfected HEK-293 cells; lane 2, HEK-293 cells transfected with rvKir6.1 cDNA; lane 3, Kir6.1C-GST fusion protein; lane 4, Kir6.2C-GST fusion protein. **B.** Normalized expression levels of Kir6.1 proteins. Column numbers bear the same meaning as the corresponding lane numbers in A. $n = 3$ for each group. * $P < 0.01$ for transfected vs. non-transfected HEK-293 cells. (This work was done by Dr. Sun as part of collaboration work; Sun et al., 2004)

A



B



Figure 3.5. Immunological reactions of different preparations to anti-GST antibody. **A.** Representative Western blots with anti-GST antibodies on cultured cells. Lane 1, non-transfected HEK-293 cells; lane 2, HEK-293 cells transfected with rvKir6.1 cDNA; lane 3, Kir6.1C–GST fusion protein (38 kDa). **B.** Representative Western blots with anti-GST antibody on different tissues. Lane 1, mesenteric artery; lane 2, tail artery; lane 3, pulmonary artery; lane 4, aorta; lane 5, heart; lane 6, liver; lane 7, spleen; lane 8, kidney; lane 9, brain; and lane 10, Kir6.1C–GST fusion protein (38 kDa) (This work was done by Dr. Sun as part of collaboration work; Sun et al., 2004)

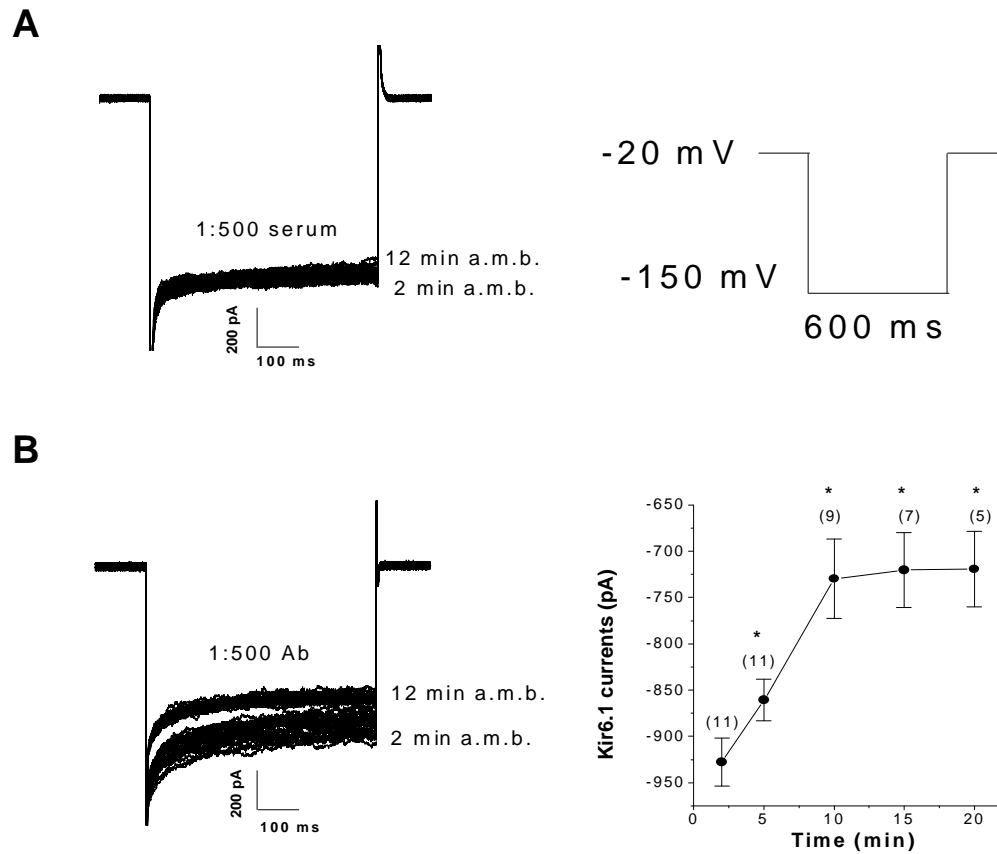


Figure 3.6. Effect of anti-Kir6.1 antibody (Kir6.1 Ab) on K_{ATP} channels in stably transfected HEK-293 cells. Test potential -150 mV; Holding potential, -20 mV. **A.** Kir6.1 currents recorded with control serum in the pipette solution. The original whole-cell K_{ATP} current traces were shown at the left and schematic representation of the depolarization protocol used for recording at the right. **B.** Kir6.1 currents recorded with Kir6.1 Ab in the pipette solution. The original whole-cell Kir6.1 current traces were shown at the left and the summary with the numbers of cells denoted at the right. The abbreviation of “a.m.b.” stands for “after membrane breaking” into the whole-cell recording configuration. * $p < 0.05$ vs. control (2 min a.m.b.).

3.3.2. Effect of nicotine on Kir6.1 channels

Nicotine at concentrations of 30 μ M increased the current densities of the inward Kir6.1 current from -23.9 ± 1.6 pA/pF to -33.3 ± 1.8 pA/pF, ($n = 6$; -150 mV; $p < 0.05$) (Fig. 3.7). When the membrane potential was set at -50 mV, nicotine at 30 μ M also caused a significant increase of Kir6.1 currents -4.0 ± 1.0 vs. -7.0 ± 1.3 pA/pF. Nicotine at 100 μ M increased Kir6.1 currents from -19.1 ± 1.6 pA/pF to -26.7 ± 4.0 pA/pF (Fig. 3.8). The maximum excitatory effect of nicotine was achieved approximately 1 min after nicotine application. The effect of nicotine was irreversible after 5-10 min of washout. Nicotine at 300 μ M had no effect on Kir6.1 currents (-14.3 ± 4.3 vs. -15.5 ± 4.4 pA/pF at -150 mV, $n = 4$, $p > 0.05$).

At 1 mM, nicotine inhibited Kir6.1 currents from -25.5 ± 5.6 to -9.9 ± 2.6 pA/pF at -150 mV ($n = 4$, $p < 0.05$). At physiological ranges of membrane potentials, nicotine also significantly inhibited Kir6.1 currents. For example, at -50 mV nicotine inhibited Kir6.1 current from -5.3 ± 0.9 to -2.4 ± 0.7 pA/pF ($p < 0.05$). This inhibitory effect of nicotine occurred gradually and lasted for 15 min in the presence of nicotine. Upon washing out nicotine from bath solution for 10 min the inhibitory effect was partially reversed (-9.9 ± 2.6 vs. -11.1 ± 6.4 pA/pF, $n = 4$, $p < 0.05$) (Fig. 3.9). The stimulatory and inhibitory effects of nicotine at different concentrations are summarized in Fig. 3.10. In non-transfected native HEK-293 cells, 30 μ M and 1 mM nicotine had no effect on the endogenous background K^+ currents either at -150 mV (-3.2 ± 1.4 vs. -5.4 ± 1.2 and -4.0 ± 0.9 vs. -4.3 ± 0.8 pA/pF, respectively) (Fig. 3.11) or at -50 mV ($-0.6 \pm$

0.2 vs. -0.9 ± 0.2 pA/pF and -0.6 ± 0.1 vs. -0.8 ± 0.1 pA/pF, respectively) ($n = 4-5$, $p > 0.05$).

To study the involvement of putative nicotinic receptors in the effects of nicotine on Kir6.1 channels, two nicotinic receptor agonists were used. Cytisine (100 μ M), an agonist of the nicotinic acetylcholine receptor (nAChR) containing $\alpha 4$ - $\beta 2$ subunits (Kihara et al., 1998), did not cause any change in Kir6.1 currents (-15.3 ± 1.4 pA/pF vs. -16.8 ± 2.5 pA/pF, testing potential -150 mV, $n = 4$, $p > 0.05$). DMPP, a non-specific nAChR agonist, also did not affect Kir6.1 currents at concentrations of 100-500 μ M. For example, after the application of 100 μ M DMPP the inward current was -14.4 ± 2.3 pA/pF at -150 mV, which was not different from the control (-16.4 ± 1.7 pA/pF, $n = 4$, $p > 0.05$) (Fig. 3.12).

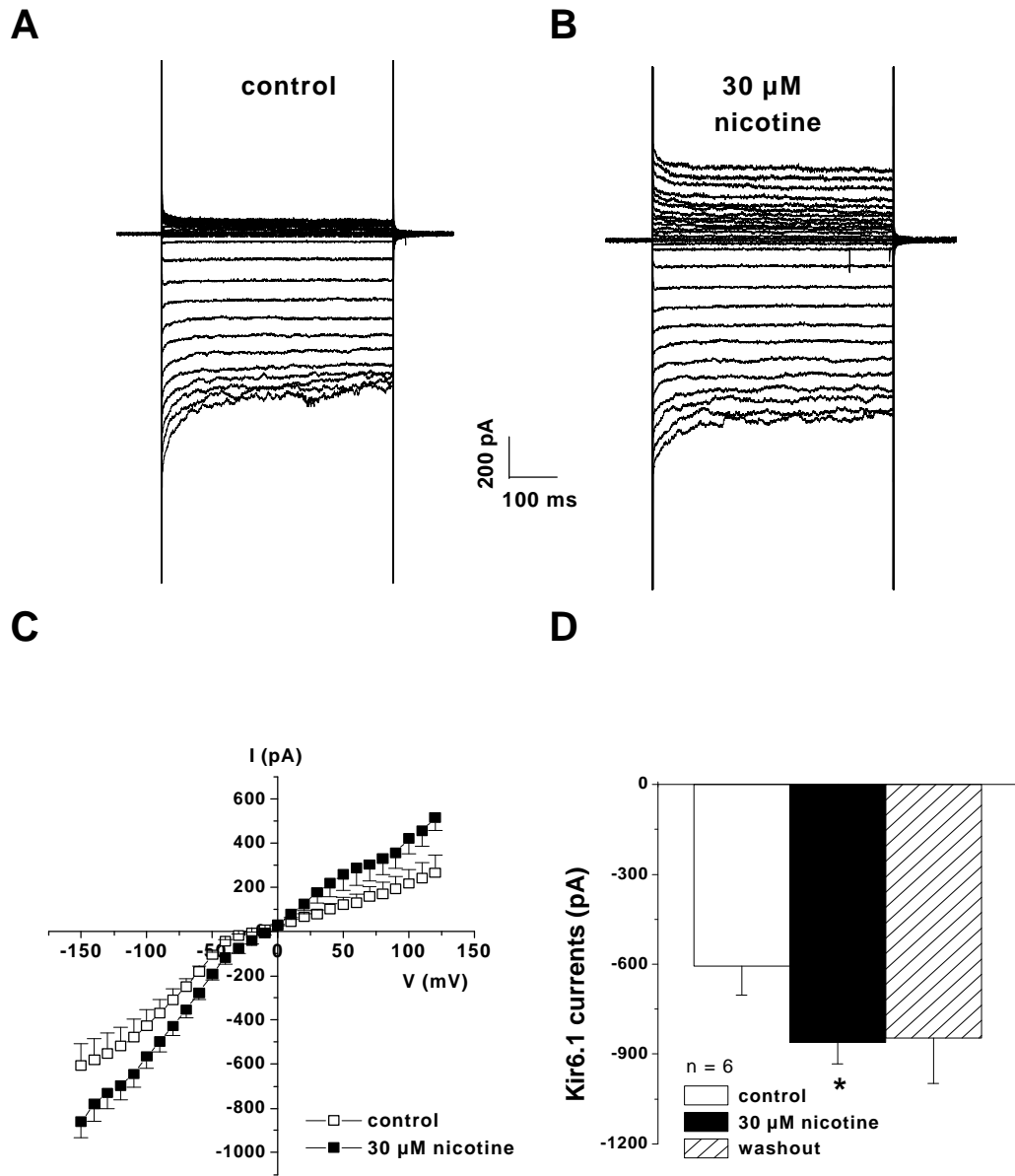


Figure 3.7. Effect of 30 μM nicotine on Kir6.1 channels. Original currents recording of Kir6.1 channels in control (A) and then application of 30 μM nicotine (B). Currents were recorded by voltage steps from -150 to +80 mV at a holding potential of -20 mV. C. I-V relationships of Kir6.1 channels before and after the application of 30 μM nicotine. D. Summary of the effect of 30 μM nicotine on Kir6.1 channels (Testing potential, -150 mV; Holding potential, -20 mV). * p < 0.05 vs. control.

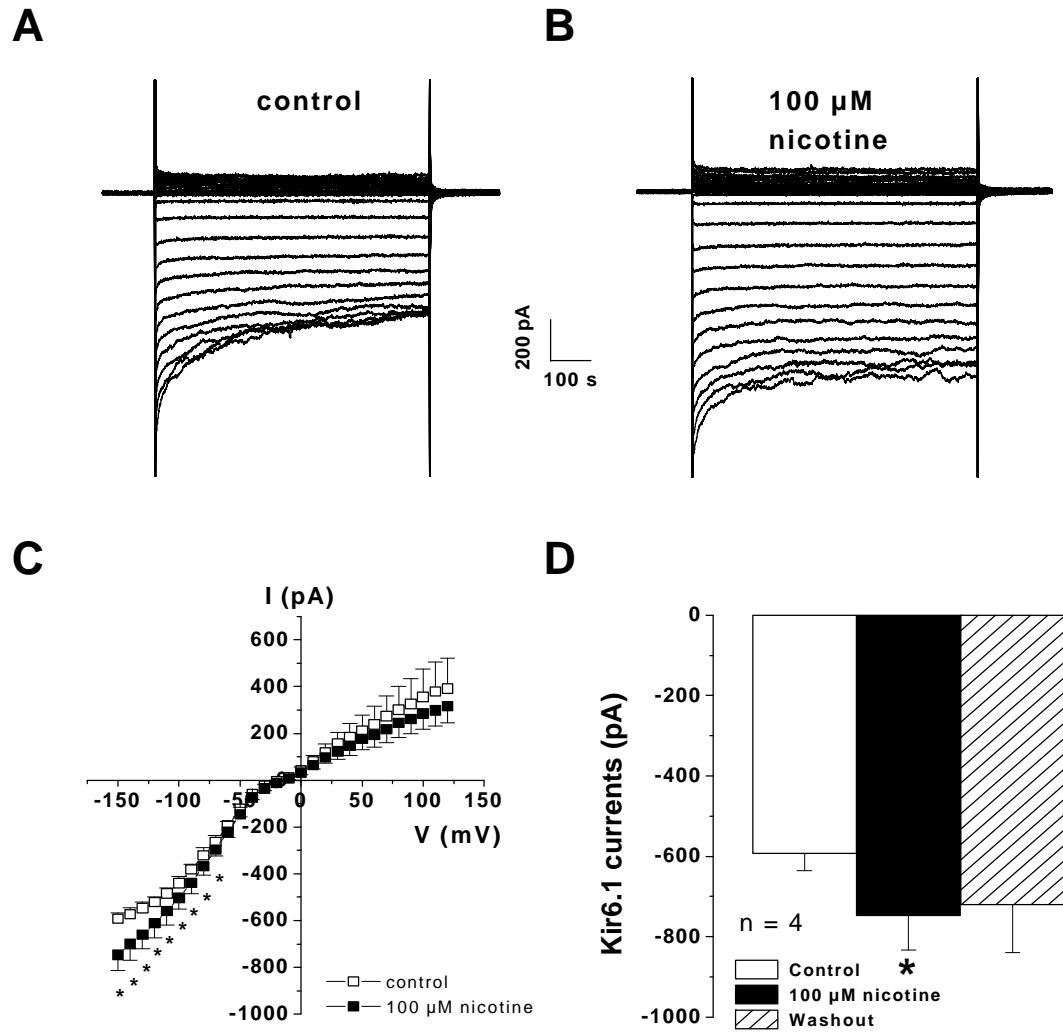


Figure 3.8. Effect of 100 μM nicotine on heterologously expressed Kir6.1 channels in HEK-293 cells. Kir6.1 currents recorded in the absence (A) and then presence of 100 μM nicotine (B). Currents were evoked by voltage steps between -150 and $+80$ mV from a holding potential of -20 mV. C. I-V relationships of Kir6.1 channels before and after the application of 100 μM nicotine. D. Summary of the effect of 100 μM nicotine on Kir6.1 channels (Testing potential, -150 mV; Holding potential, -20 mV). * $p < 0.05$ vs. control.

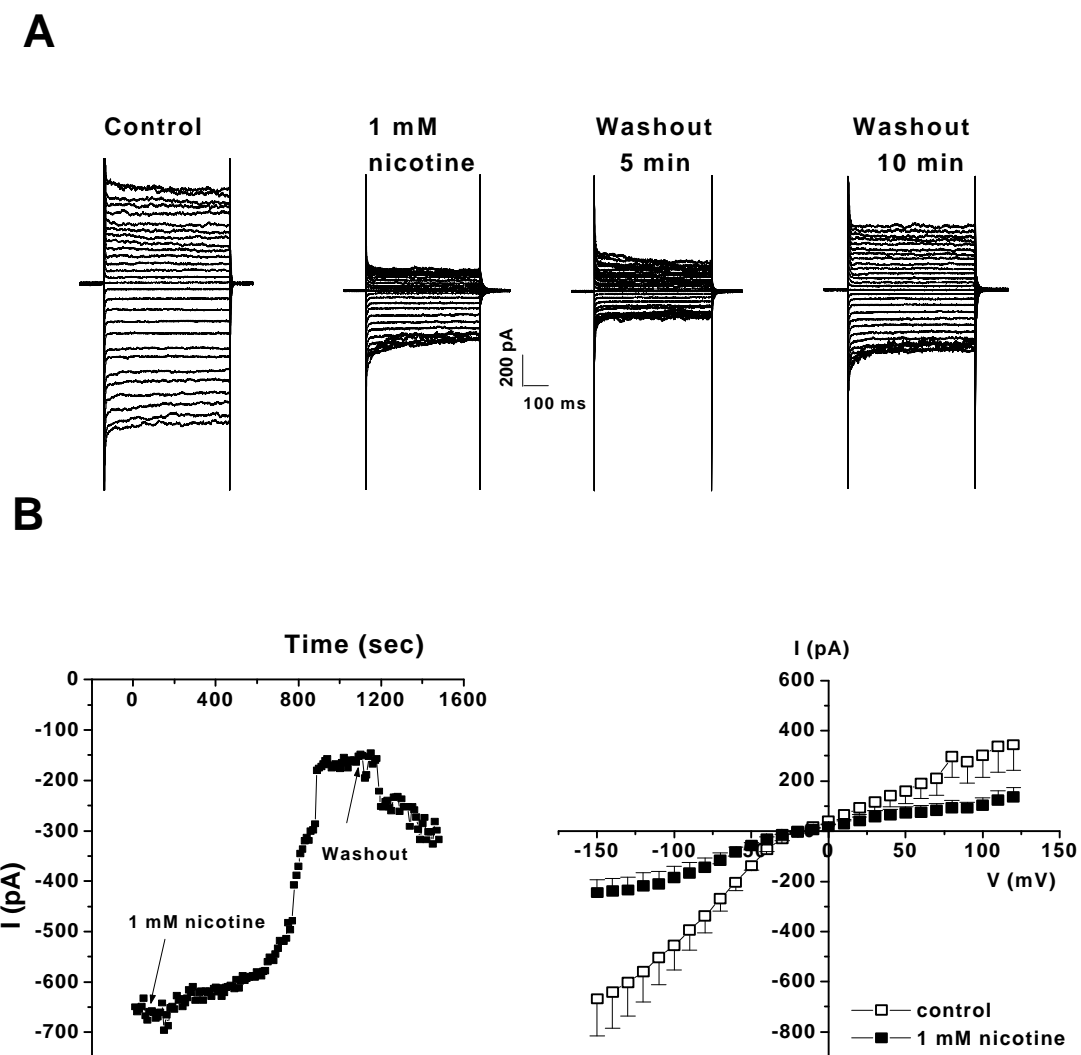


Figure 3.9. Effect of 1 mM nicotine on Kir6.1 channels. Representative original cell recording in control, after application of 1 mM nicotine (**A**) and during washout (**B**). **C.** Representative time course of the effect of 1 mM nicotine on Kir6.1 channels (Testing potential, -150 mV; Holding potential, -20 mV). **D.** I-V relationship of Kir6.1 currents before and after application of nicotine. * $p < 0.05$ vs. control.

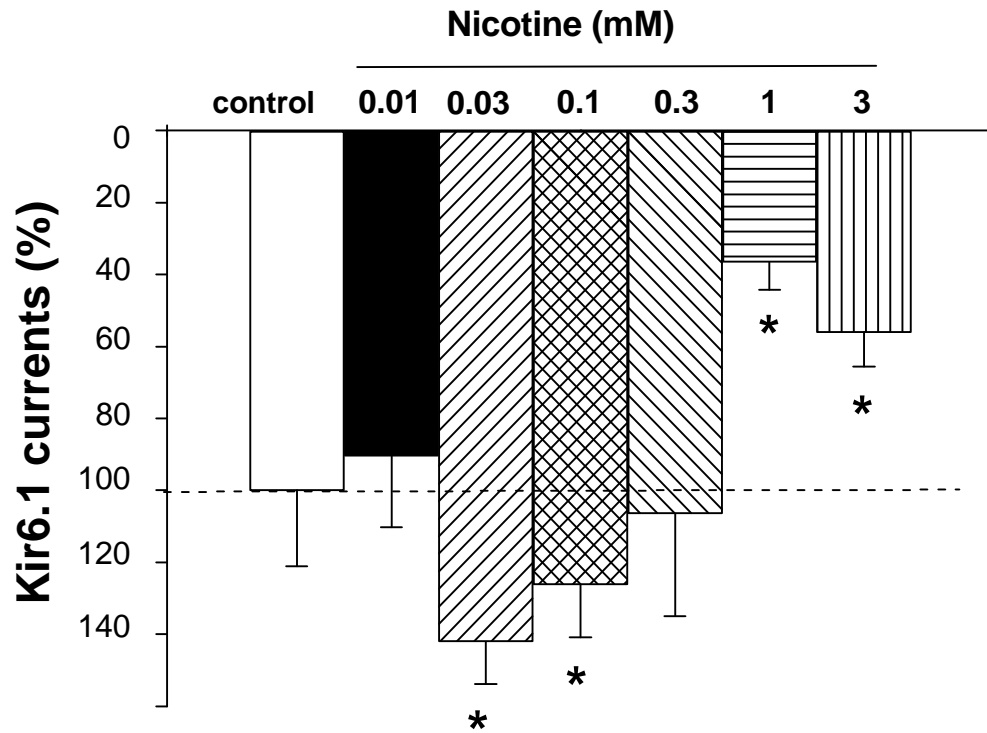


Figure 3.10. Summary of the effect of nicotine on Kir6.1 channels. Relative changes in mean Kir6.1 currents (at -150 mV) induced by different concentrations of nicotine. Holding potential, -20 mV. * $p < 0.05$ vs. control.

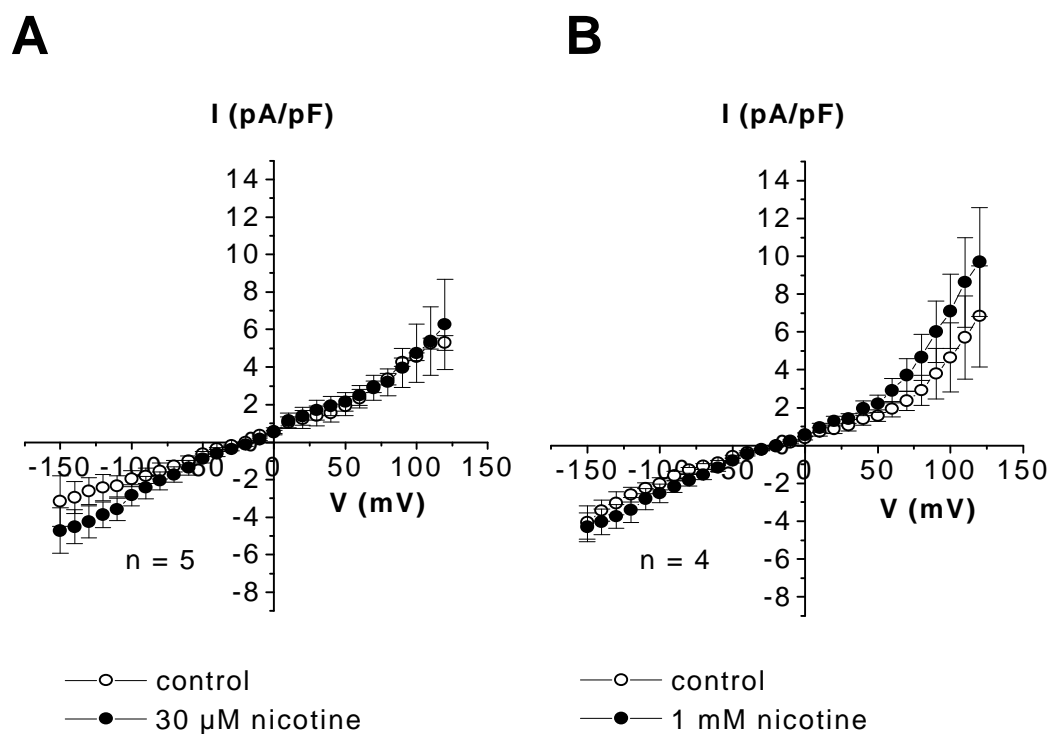


Figure 3.11. Effect of nicotine on endogenous whole-cell currents in non-transfected HEK-293 cells. I-V relationships of native transmembrane currents before and after the application of nicotine at 30 μ M (**A**) and 1 mM (**B**) were shown.

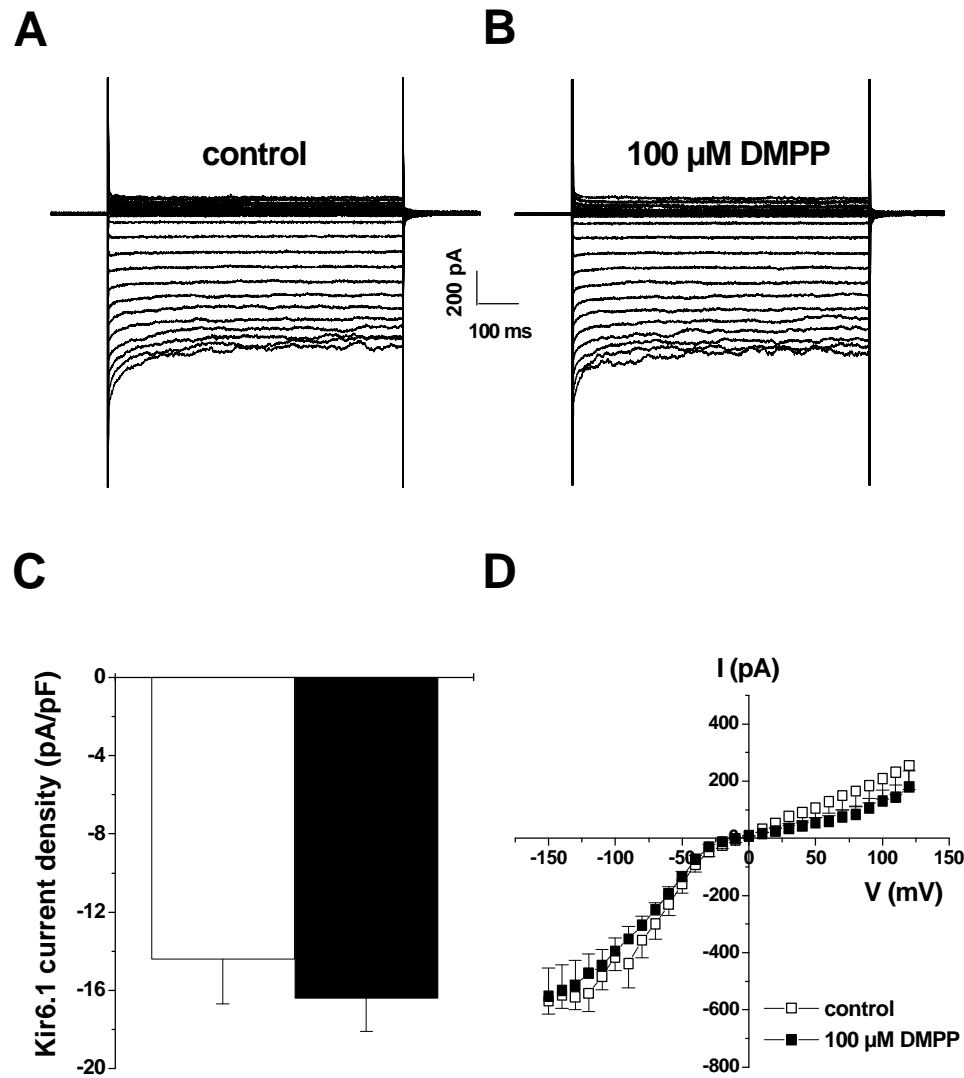


Figure 3.12. Effect of DMPP on Kir6.1 channels stably expressed in HEK-293 cells. Original recordings of Kir6.1 currents before (A) and after (B) the application of 100 μM DMPP to the bath solution. C. Summary of the effects of DMPP on Kir6.1 channels at -150 mV. D. I-V relationship of Kir6.1 channels before and after the application of DMPP. * $p < 0.05$.

3.3.3. Effect of nicotine on Kir6.1 channels in the presence of Kir6.1 Ab in the pipette solution

After the inhibitory effect of Kir6.1 Ab on Kir6.1 channels became stable (Fig. 3.13A), the cells were exposed to 100 μ M nicotine, which only induced a 3.2% increase in Kir6.1 currents ($n = 7$, $p > 0.05$) (Fig. 3.13B & C). Similarly, dialysis with 1:500 Kir6.1 Ab in the pipette solution abolished the inhibitory effect of 1 mM nicotine on Kir6.1 current (Fig. 3.14).

3.3.4. Nicotine-induced production of superoxide anion

In HEK-293 cells permanently transfected with Kir6.1 cDNA, nicotine (100 μ M) increased the production of $O_2^{\cdot -}$ from 24.6 ± 1.4 to 33.0 ± 1.6 cpm/ 10^6 cells ($n = 6$, $p < 0.05$). On the other hand, 1 mM nicotine caused a decrease in the production of $O_2^{\cdot -}$ from 24.6 ± 1.4 to 9.0 ± 0.5 cpm/ 10^6 cells ($n = 6$, $p < 0.01$). In the presence of tempol (100 μ M), the production of $O_2^{\cdot -}$ induced by 100 μ M and 1 mM nicotine was decreased to 22.8 ± 1.1 and increased to 11.6 ± 0.5 cpm/ 10^6 cells, respectively ($n = 12$) (Fig. 3.15A). In non-transfected HEK-293 cells, nicotine at 100 μ M increased the production of $O_2^{\cdot -}$ from 9.4 ± 0.2 to 13.5 ± 0.4 cpm/ 10^6 cells ($n = 5-8$ for each group, $p < 0.05$). Nicotine at 1 mM inhibited $O_2^{\cdot -}$ production from 9.4 ± 0.2 to 4.6 ± 0.1 cpm/ 10^6 cells ($n = 5-8$ for each group, $p < 0.05$) (Fig. 3.15B). There was a significant difference in the basal level of $O_2^{\cdot -}$ production between transfected (24.6 ± 1.4 cpm/ 10^6 cells) and non-transfected HEK-293 cells (9.4 ± 0.2 cpm/ 10^6 cells) ($n = 5-8$ for each group, $p < 0.05$).

3.3.5. Effects of HX/XO on Kir6.1 channels

The HX/XO reaction was used as a source of O_2^- . When HX (100 μ M) alone was added to the bath solution, no detectable changes in Kir6.1 currents were observed in any of the cells tested. Application of XO (60 mU/ml) in the presence of HX significantly increased the amplitude of Kir6.1 currents. The stimulatory effect of HX/XO on Kir6.1 channels occurred within 30 s of application and lasted as long as the cells were exposed to HX and XO (Fig. 3.16A). A complete recovery of Kir6.1 currents to the control level required about 5 min of washout after the first time of the application of HX/XO. Kir6.1 channels responded well to consecutive HX/XO stimulations (Fig. 3.16A). Recovery of Kir6.1 currents again required 5 min of washout after the second time application of HX/XO. The stimulatory effect of HX/XO on Kir6.1 current was blocked by 100 μ M tempol prior to the application of HX/XO to the bath solution (Fig. 3.16B). The stimulatory effect of 30 μ M nicotine on Kir6.1 current was abolished by prior application of 100 μ M tempol to the bath solution (-28.3 ± 6.1 pA/pF vs. -31.2 ± 7.3 pA/pF at -150 mV, $n = 6-9$ for each group, $p > 0.05$) (Fig. 3.16C).

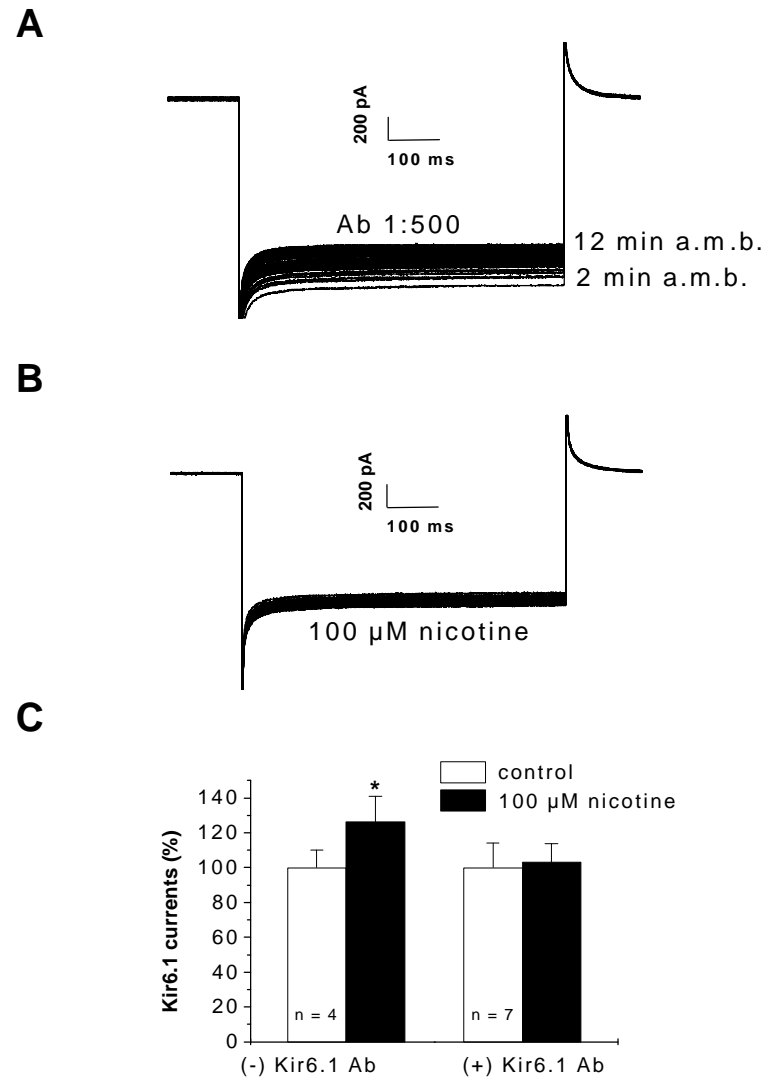


Figure 3.13. The stimulatory effects of nicotine on Kir6.1 channels in the presence of anti-Kir6.1 antibody (Kir6.1 Ab) in the pipette solution. **A.** Time-dependent inhibition of Kir6.1 channel by Kir6.1 Ab. **B.** Kir6.1 currents in the same cell as in A, which was subsequently exposed to 100 μ M nicotine. **C.** Comparison of the effects of 100 μ M nicotine in the absence and presence of Kir6.1 Ab. * $p < 0.05$ vs. control. Kir6.1 currents were recorded at -150 mV from a holding potential of -20 mV. The abbreviation of “a.m.b.” stands for “after membrane breaking” into the whole-cell recording configuration.

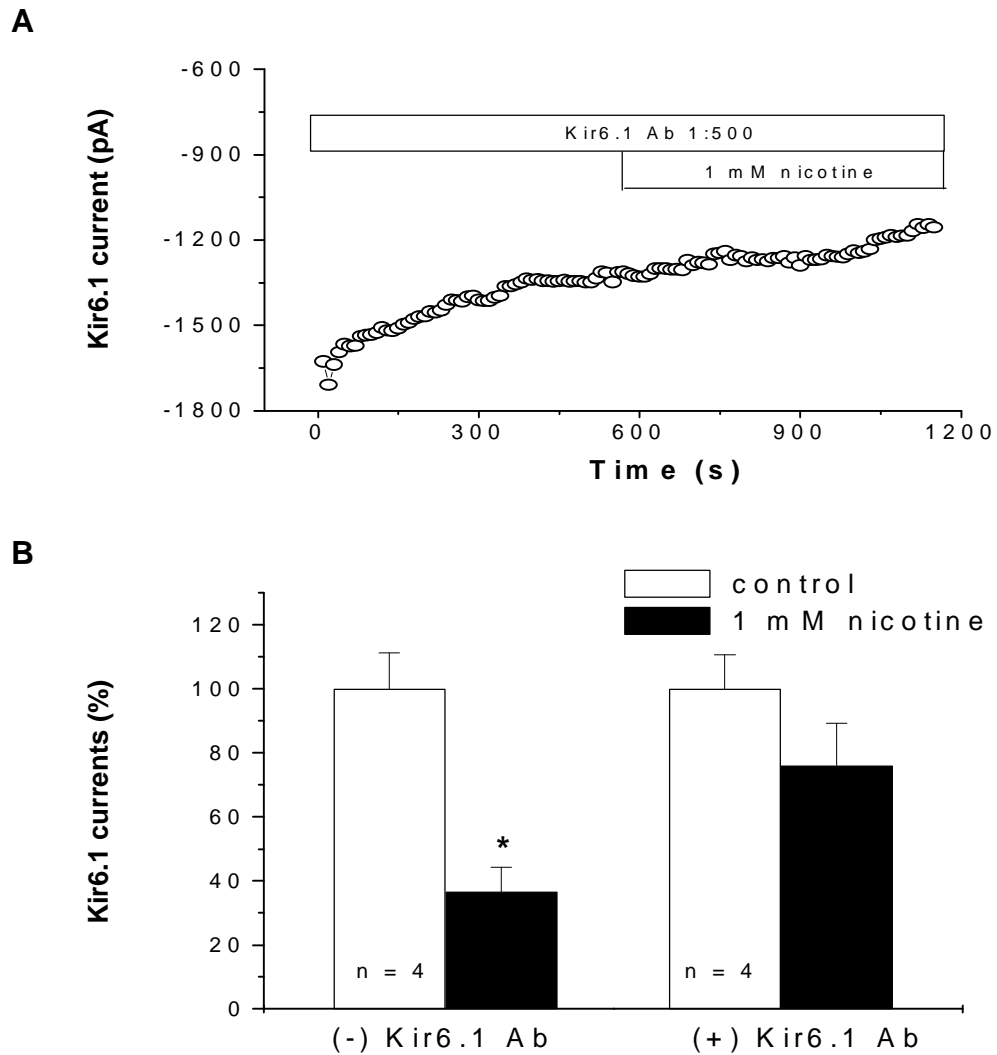


Figure 3.14. The inhibitory effects of nicotine on Kir6.1 channels in the presence of anti-Kir6.1 antibody (Kir6.1 Ab). **A.** The time-dependent changes in Kir6.1 current with Kir6.1 Ab in the pipette solution and 1 mM nicotine in the bath solution. **B.** Comparison of the effects of 1 mM nicotine in the absence and presence of Kir6.1 Ab in the pipette solution. * $p < 0.05$ vs. control. Kir6.1 currents were recorded at -150 mV from a holding potential of -20 mV.

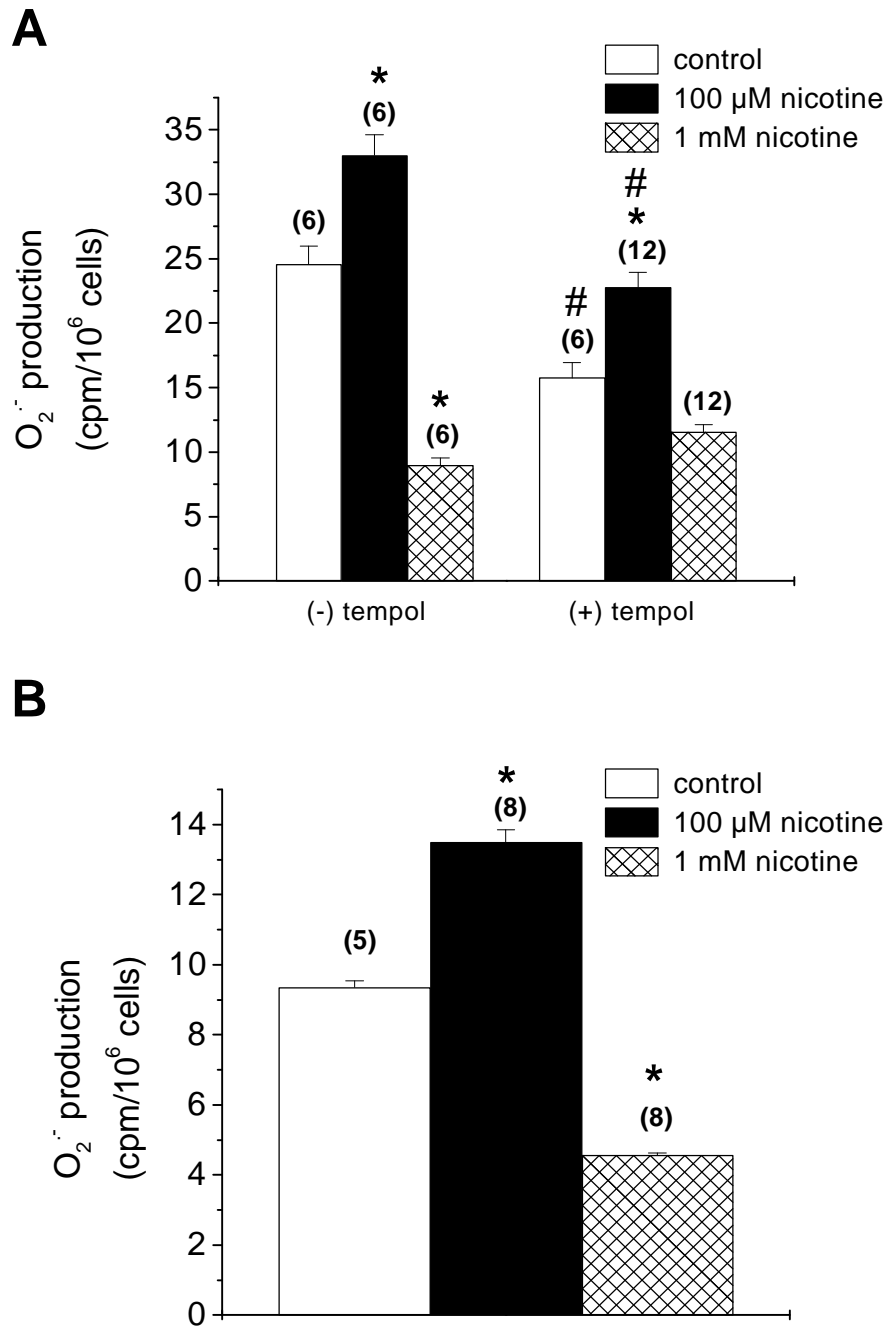


Figure 3.15. O₂⁻ production in HEK-293 cells permanently transfected with Kir6.1 cDNA (**A**) or in non-transfected HEK-293 cells (**B**). * p<0.05 vs. control in the same group (with or without tempol). # p<0.05 vs. the same treatment in the absence of tempol. Numbers of experiments for each treatment are denoted.

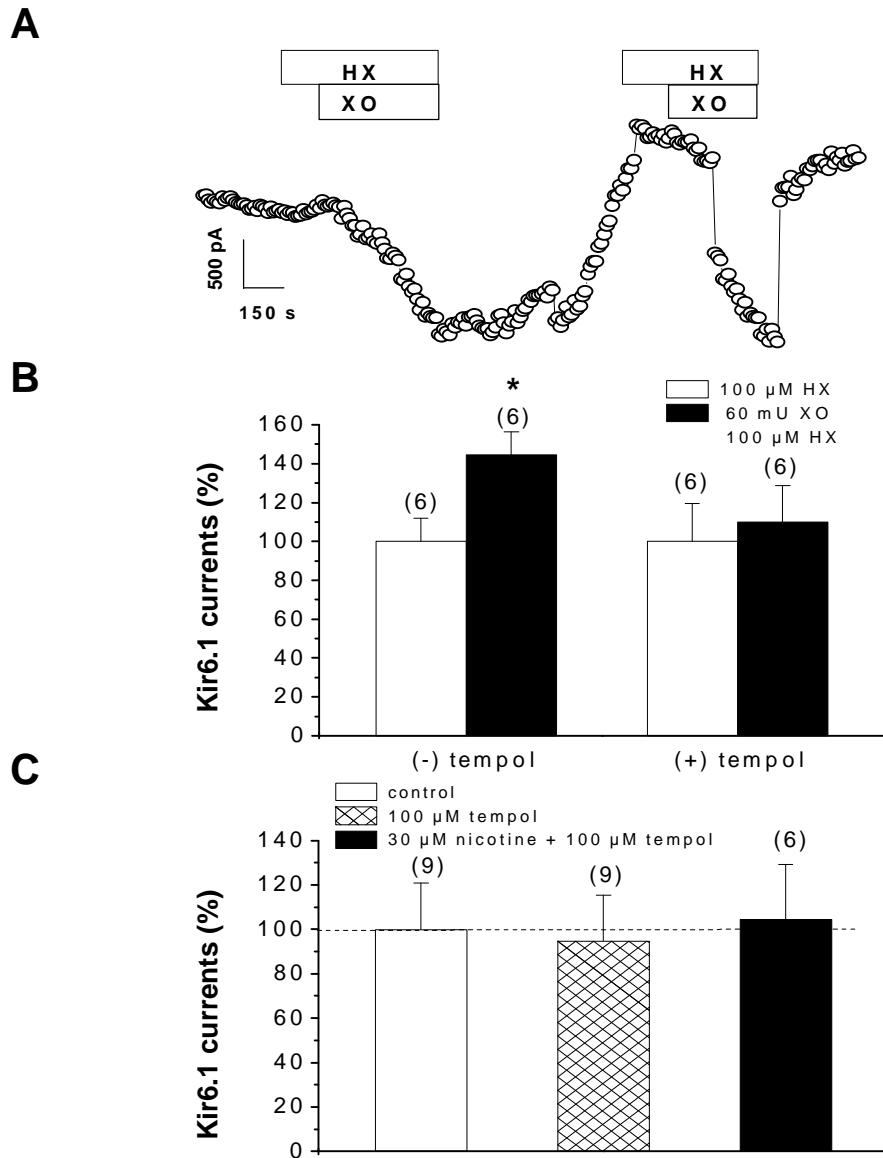


Figure 3.16. Modulatory effect of $O_2^{\cdot -}$ on Kir6.1 channels. **A.** Representative current records showing changes in Kir6.1 currents in HEK-293 cells induced by sequentially applied HX (100 μ M) and XO (60 mU/ml). **B.** Summary of the effects of HX and XO on Kir6.1 channels in the presence and absence of tempol. $n = 6$. * $p < 0.05$ vs. 100 μ M HX. **C.** Summary of the effects of nicotine (30 μ M) on Kir6.1 channels in HEK-293 cells in the presence of tempol (100 μ M). Numbers of cells in each group are denoted.

3.4. DISCUSSION

The effects of nicotine on ion channels have only been studied in a few cases. Wang et al. (2000a) reported that nicotine potently blocked cardiac A-type channels and significantly delayed canine ventricular repolarization independent of the selected nAChR or mAChR. In cultured rat striatal neurons, nicotine inhibited slowly inactivating K^+ currents (Hamon et al., 1997). This neuronal effect of nicotine was attributed to the stimulation of nicotine receptors because the nicotinic antagonists reversed, and nicotinic agonists reproduced the ion channel blockade. Previous studies from our laboratory showed that in rat tail artery SMCs nicotine differentially affect the whole-cell K^+ channel currents (Tang et al., 1999). At low concentrations (1-100 μ M), nicotine increased K^+ channel currents via the stimulation of nicotine receptors. At high concentrations (0.3-3 mM), nicotine inhibited K^+ currents due to its direct effect on K^+ channel proteins since the inhibitory effect of nicotine cannot be abolished by nicotinic antagonist. These observations on isolated vascular SMCs were reconciled by a vascular contractility study. Nicotine induced three types of vasoactive responses of isolated rat tail artery tissues, i.e., contraction, relaxation, and rebound contraction upon the removal of nicotine (Wang and Wang, 2000). Nicotine caused a transient contraction of rat tail artery strips in an extracellular Ca^{2+} dependent and endothelium independent fashion. Incubating tail artery tissues with the nicotine receptor antagonist dihydro- β -erythroidine hydrobromide at 10 μ M did not affect the nicotine-induced vasoconstriction, vasorelaxation, or the nicotine withdrawal induced rebound contraction. When the concentration of dihydro- β -erythroidine hydrobromide was increased to 300 μ M, the nicotine-induced vasodilatation, but not the vasoconstriction or the nicotine withdrawal

induced rebound contraction, was significantly reduced. Nicotine can also exert its cellular effects without the involvement of nAChRs or catecholamine release. Several studies performed under conditions devoid of nAChR stimulation demonstrated the ability of nicotine to alter action potential characteristics in various species including guinea pig (Pappano and Rembush, 1970) and rabbit (Carryl et al., 1992), as well as in different types of tissues such as sinus node (Satoh, 1997), atrium, ventricle, and Purkinje fiber. This present study is the first one reporting the effects of nicotine on the cloned Kir6.1 channel protein. Nicotine at low concentrations (30-100 μ M) immediately increased, but at millimolar concentrations gradually inhibited, Kir6.1 currents. The presence of Kir6.1 Ab abolished both the inhibitory and stimulatory effects of nicotine at different concentrations on Kir6.1 currents. Since the Kir6.1 Ab was against a 79 aa fragment of Kir6.1 at its C terminus (Sun et al., 2004), the interaction of nicotine with Kir6.1 channels in the presence of Kir6.1 Ab suggest that this 79 aa C-terminal epitope may be the interacting sites of Kir6.1 channel protein with nicotine.

Cytisine specifically stimulates the $\alpha 4$ - $\beta 2$ nAChR (Kihara et al., 1998) and DMPP induces the non-specific stimulation of nAChRs. These two agonists did not have any effect on Kir6.1 currents at the concentrations tested. Northern blot and Western blot analysis have shown the absence of nAChRs in native HEK-293 cells (Chavez-Noriega et al., 2000). Based on these observations, it was concluded that the interaction of nicotine with Kir6.1 channels is independent of nicotine receptors.

Previous evidence suggests that PNU-37883A, in contrast to other K_{ATP} channel modulators, does not bind to the SUR subunit (Kovalev et al., 2004). PNU-37883A and glyburide works synergistically to inhibit K^+ channel opener-mediated vasorelaxation of

isolated rabbit mesenteric artery (Ohrnberger et al., 1993). PNU-37883A does not affect pinacidil binding to rat aorta and only blocks glyburide binding nonspecifically at high concentration (Loffler-Walz and Quast, 1998). There is a considerable body of pharmacological and physiological data that suggest that PNU-37883A selectively interacts at the vascular but not pancreatic K_{ATP} channels. 3H -PNU-37883A showed specific, displaceable binding to rabbit mesenteric artery but not to pancreatic rat insulin-secreting RINm5F insulinoma cell membranes (Guillemare et al., 1994). In a more comprehensive electrophysiological analysis, Wellman et al. (1999) showed that PNU-37883A inhibited pinacidil-activated K_{ATP} currents in single rat mesenteric artery SMCs, but had little effect on those elicited in skeletal or cardiac myocytes. Since vascular K_{ATP} channels are widely held to be comprised of the Kir6.1 and SUR2B subunits, the selective effect of PNU-37883A suggest that Kir6.1 may be the target of this compound. Consistent with this notion, Kovalev et al. (2004) have assigned the PNU-37883A sensitivity to a carboxyl-terminal region of Kir6.1 (amino acids 200-424). Only chimeras including this carboxyl-terminal protein of Kir6.1 show high sensitivity to this compound. They have also demonstrated that the region between 200 and 280 amino acids plays a key role in determining sensitivity to PNU-37883A. Our cloned full-length Kir6.1 channel cDNA contains the essential amino acids (amino acids 200-424) as a site of action for PNU-37883A. In our experimental conditions, PNU-37883A inhibited Kir6.1 currents by 30.9%. Cui et al. (2003) reported 52.4% inhibition for Kir6.1/SUR2B by 30 μM PNU37883A. Kovalev et al. (2004) reported around 20% inhibition of Kir6.1/SUR2B by 10 μM PNU-37883A, consistent with the effect of PNU-37883A reported in the present study. Four lines of evidence support the conclusion that the recorded inward current in our transfected HEK-293 cells is conducted through

Kir6.1 channel. First, in our previous published paper (Sun et al., 2004) using Western blot experiment we detected a single 41 kDa band of Kir6.1 protein in HEK-293 cells that were permanently transfected with Kir6.1. The application of anti-Kir6.1 antibody specifically recognized a single 38 kDa bacterial Kir6.1C-GST fusion protein and this specific antibody did not cross-react with a Kir6.2C-GST fusion protein. After the anti-Kir6.1 antibody was pre-absorbed with Kir6.1 bacterial fusion protein prior to Western blot experiments, positive bands in HEK-293 cells disappeared. Second, the electrophysiological recordings demonstrated that K_{ATP} channels in transfected HEK-293 cells were significantly inhibited by the anti-Kir6.1 antibody. The inhibitory effect of anti-Kir6.1 antibody was specific since the recorded K_{ATP} currents did not change in amplitude over the same time frame in the absence of the antibody in the pipette solution or with the inclusion of control serum. Third, PNU-37883A significantly inhibited Kir6.1 currents.

The nicotine-induced endothelium-dependent vasodilatation was reversed by topical application of SOD (Mayhan and Sharpe, 1998). Modulation of K^+ channel activity by oxidative stress is important for cellular functions. $O_2^{\cdot -}$ have been reported to increase K_{ATP} channel activity in guinea-pig cardiac myocytes but decrease K_{ATP} channel opening in cerebral vasculature (Goldhaber et al., 1989; Tokube et al., 1996). Both hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) enhance K_{ATP} channel activity in the myocardium (Ichinari et al., 1996) and in the coronary (Liu et al., 1994), renal, mesenteric (Benkusky et al., 1998) and cerebral vascular beds (Wei et al., 1996; 1998).

Our study suggests that $O_2^{\cdot -}$ may directly stimulate the Kir6.1 channel. Adenine nucleotides are metabolized to HX and xanthine. The nicotinamide adenine dinucleotide

(NAD⁺)-reducing enzyme xanthine dehydrogenase is converted to XO *in vivo* by various mechanisms. Involvement of XO in aggravating oxidative stress seems to involve ischemia, when xanthine dehydrogenase is converted into xanthine oxidase, which then releases superoxide. The conversion of xanthine dehydrogenase to XO is thought to be triggered by the accumulation of large amounts of xanthine that accumulates during ischemia because of the breakdown of ATP. The reaction between HX and XO generates O₂⁻, H₂O₂, and uric acid. In our patch-clamp study, HX alone did not alter Kir6.1 currents and co-application of HX and XO produced a significant increase in Kir6.1 currents in all cells tested. This stimulatory effect was abolished by tempol, a scavenger of O₂⁻. A causative relationship between O₂⁻ and Kir6.1 channel function is therefore strongly indicated.

At micromolar concentrations, nicotine enhanced superoxide production (Fig. 3.15). This may explain the stimulatory effect of nicotine on Kir6.1 channels since superoxide anion directly stimulated the Kir6.1 channel (Fig. 3.16A & B) and tempol abolished the stimulatory effect of nicotine on Kir6.1 channels (Fig. 3.16C). On the other hand, nicotine at millimolar concentrations inhibited basal superoxide production (Fig. 3.15). A lower basal level of superoxide anion can be linked to a reduced stimulation on Kir6.1 channels. Thus, this would underline the inhibitory effect of nicotine on Kir6.1 channels. Whether nicotine can have similar effects on Kir6.2 subunits is currently unknown since Kir6.2 is incapable of independent expression in HEK-293 cells (Ammala et al., 1996b).

In summary, our study demonstrated that nicotine at micromolar concentrations stimulated, while at millimolar concentrations inhibited Kir6.1 currents. The dual effects of nicotine, not mediated by nAChR, are mediated by superoxide levels in the cells. Not

only HX/XO-generated $O_2^{\cdot -}$ stimulates Kir6.1 channels, scavenging $O_2^{\cdot -}$ with tempol also abolished the effects of HX/XO as well as nicotine on Kir6.1 channels. Furthermore, significant production of $O_2^{\cdot -}$ by nicotine was demonstrated. The results of the present study have several important implications. (1) Oxidative stress related to nicotine consumption may underlie some cellular changes induced by smoking and/or smokeless tobacco usage. (2) Superoxide anions may alter cellular functions by interacting with Kir6.1 channels. Further studies using these lines of information will help to better understand the modulation mechanisms of K_{ATP} channels.

4. INTERACTION OF ACh WITH KIR6.1 CHANNELS HETEROLOGOUSLY EXPRESSED IN HEK-293 CELLS

4.1. INTRODUCTION

Acetylcholine (ACh) is a major neurotransmitter found in parasympathetic nerve terminals and its effects on cardiac function are generally inhibitory via activation of myocardial muscarinic receptors. There are two important types of parasympathetic (cholinergic) receptors. Nicotinic receptors are found mainly in skeletal muscle and ganglial cells. Muscarinic receptors have quite different structures from those of nicotinic receptors. Muscarinic receptors are encoded by a number of different genes; most important are the M1-subtype, found mainly in autonomic ganglia and the central nervous system, M2-subtype in the heart, and M3-subtype in smooth muscle and secretory cells. All of these muscarinic receptors are blocked by atropine.

ATP-sensitive K^+ channels (K_{ATP} channels) play a crucial role in coupling the cell's metabolic status to its membrane potential, thereby functioning as cellular metabolic sensors (Ashcroft and Ashcroft, 1990). K_{ATP} channels are regulated by intracellular ADP- and ATP-concentrations (Sun et al., 2004; Babenko et al., 1998) and plasma membrane phospholipid (Baukrowitz and Fakler, 2000). In addition, diazoxide activates the channels, and sulphonylureas like tolbutamide and glibenclamide inhibit channel activity (Kozlowski et al., 1989). Based on their central position in metabolic and signaling pathways, potassium channels are increasingly recognized as potential targets for pharmacological intervention (Sun et al., 2004). One of the best examples of this strategy is the successful therapy with sulphonylureas acting on K_{ATP} channels in

pancreatic beta-cells, increasing their insulin output, and improving the impaired regulation of glucose metabolism in diabetic patients (Inagaki and Seino 1998).

K_{ATP} channels have an important role in the regulation of cellular functions. However, there is no electrophysiological or pharmacological study reporting the effects of ACh on K_{ATP} channels.

4.1.1. Objectives of the study

The first goal of the present study was to examine the effect of ACh on the cloned Kir6.1 subunit from rat mesenteric artery SMCs, which was heterologously expressed in HEK-293 cells. The second goal was to investigate whether the effect of ACh was mediated by the traditional pathways of muscarinic receptor stimulation.

4.2. MATERIALS AND METHODS

4.2.1. Stable transfection of HEK-293 cells with Kir6.1 gene

Details of the stable transfection are described in section 2.1.

4.2.2. Electrophysiological recording of the whole-cell K_{ATP} channel currents

The whole cell patch-clamp technique was used to record Kir6.1 currents. Details of these methods are described in section 2.2.

4.2.3. Data analysis

Details of the statistical analysis are described in section 2.5.

4.3. RESULTS

4.3.1. Effect of ACh on Kir6.1 channels

The properties of the expressed Kir6.1 channels in HEK-293 cells and the endogenous background K⁺ channel currents were discussed in section 3.3.1. We used Kir6.1 Ab to identify the expressed current. Kir6.1 Ab significantly inhibited Kir6.1 channels (Fig. 4.1A). The inhibitory effect of anti-Kir6.1 antibody was specific since the recorded Kir6.1 currents did not change their amplitude over the same time frame in the absence of the antibody in the pipette solution or with the inclusion of control serum (Fig. 4.1B).

In the presence of 0.3 mM ATP in the pipette solution, ACh at 10 μ M increased Kir6.1 current density from -22.4 ± 4.7 to -36.9 ± 5 pA/pF at -150 mV ($n = 5$, $p < 0.05$). The effect of ACh became even greater when the ACh concentration was decreased to 1 μ M ($n = 8$, $p < 0.05$) (Fig. 4.2A, B). There was a concentration dependent relationship for the stimulatory effect of ACh at 1, 10 and 100 μ M (109.6 ± 37.7 %, 66.6 ± 29.5 %, and 36.7 ± 15.3 %) (Fig. 4.2C). In non-transfected HEK-293 cells ACh had no effect on the background currents (-4.0 ± 1.1 vs. -4.0 ± 1.4 pA/pF, $n = 5$, $p > 0.05$) (Fig. 4.3).

4.3.2. Effect of intracellular ATP concentrations on Kir6.1 channels

To determine whether the ACh stimulatory effect was affected by intracellular ATP concentration, we increased the ATP concentration in the pipette solution to 5 mM. Kir6.1 currents were smaller, although not significantly, with 5 mM ATP in the pipette solution in comparison with the currents with 0.3 mM ATP (-16.4 ± 2.3 vs. -22.4 ± 4.7

pA/pF, $n = 8-5$, $p > 0.05$). ACh at 10 μM still exhibited its stimulatory effect on Kir6.1 channels (-16.4 ± 2.3 to -25.5 ± 3.8 pA/pF, $n = 8$, $p < 0.05$) (Fig. 4.4).

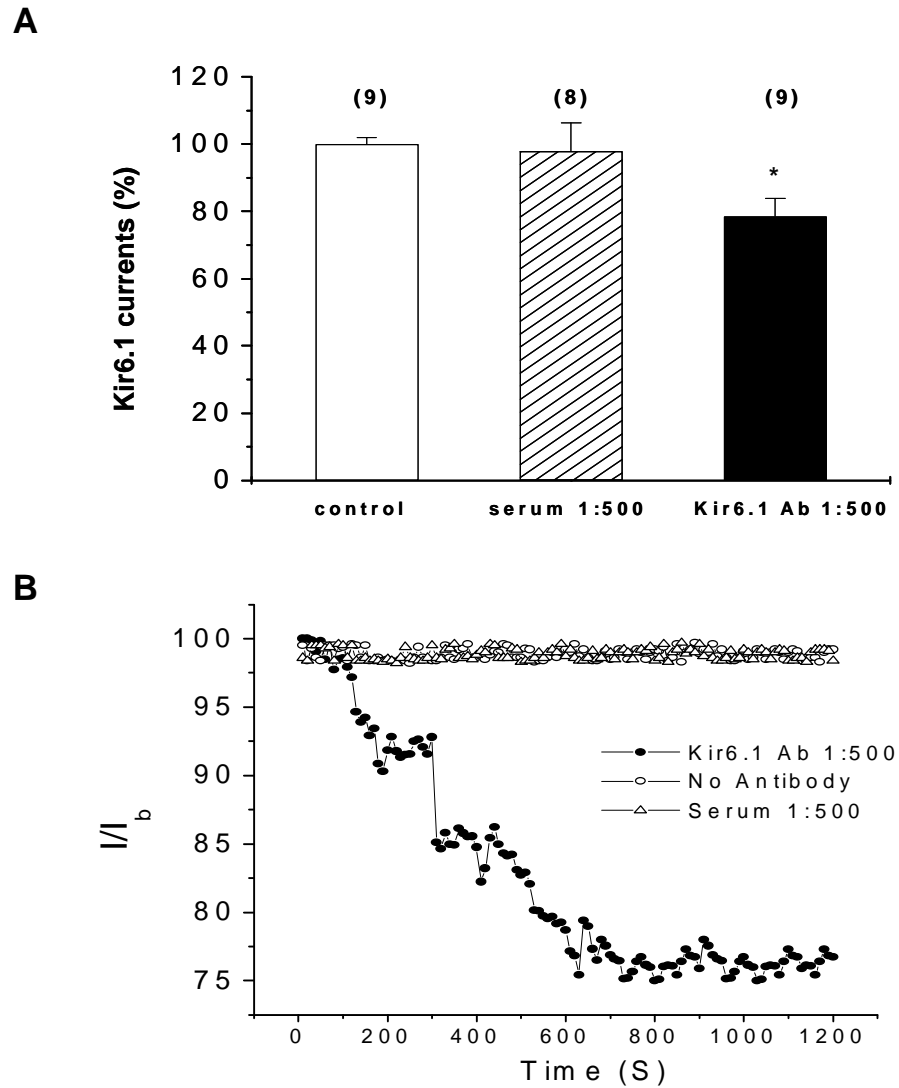


Figure 4.1. Effect of anti-Kir6.1 antibody (Kir6.1 Ab) on Kir6.1 currents. **A.** Effect of Kir6.1 Ab on Kir6.1 channels in stably transfected HEK-293 cells. Test potential, -150 mV; Holding potential, -20 mV. Kir6.1 currents recorded with control serum or Kir6.1 Ab in the pipette solution. **B.** The time-dependent effect of Kir6.1 Ab on whole-cell K_{ATP} currents. Current amplitude (I) at -150 mV at each time point was compared with those obtained at the beginning of the recording (I_b). Holding potential -20 mV. * $p < 0.05$ vs. control. Number of cells for each experiment is denoted.

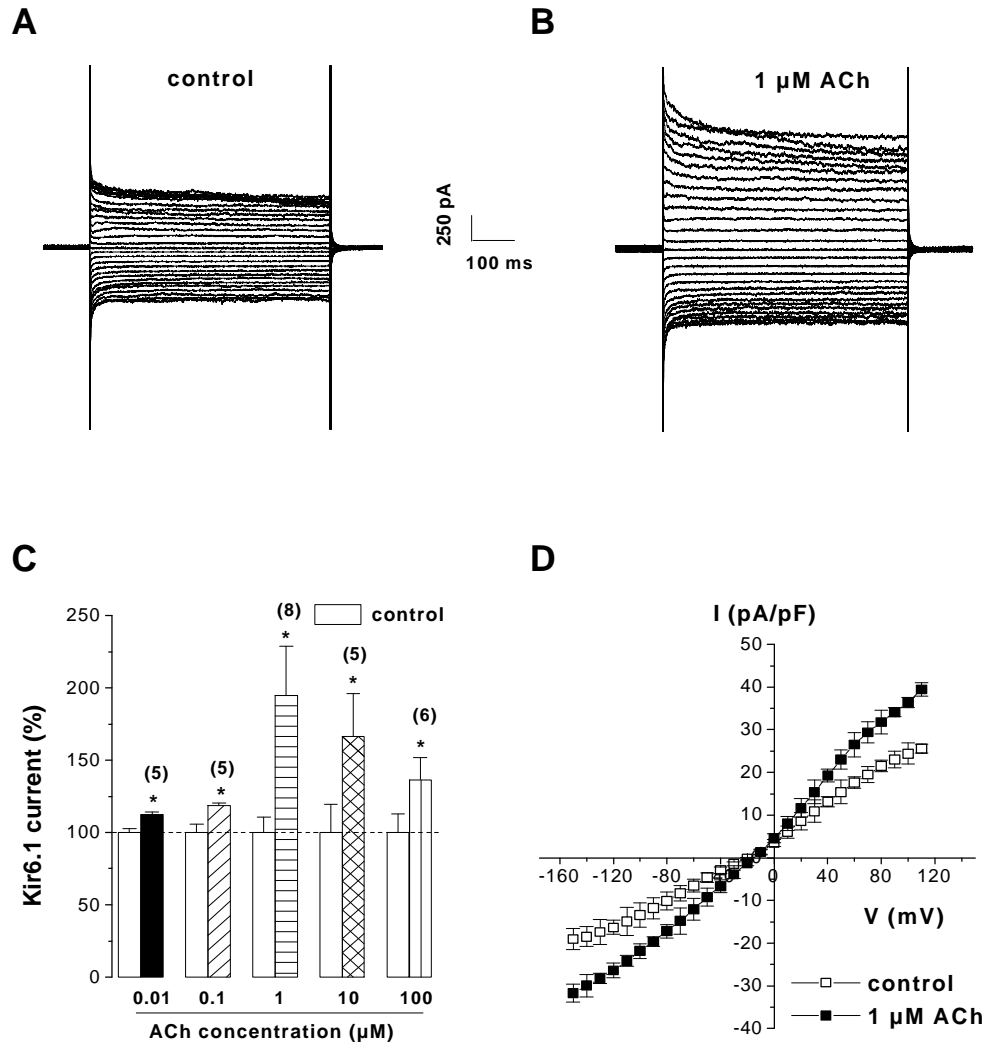


Figure 4.2. Effect of ACh on heterologously expressed Kir6.1 channels in HEK-293 cells. Kir6.1 currents recorded in the absence (**A**) and then presence (**B**) of 1 μM ACh. Currents were evoked by voltage steps from -150 to $+80$ mV with a holding potential of -20 mV. **C.** Normalized changes in mean Kir6.1 currents (at -150 mV) induced by different concentrations of ACh. * $p < 0.05$ vs. control. Numbers of cells for each experiment are denoted. **D.** I-V relationships of Kir6.1 channels before and after the application of 1 μM ACh. Holding potential, -20 mV; $n = 8$.

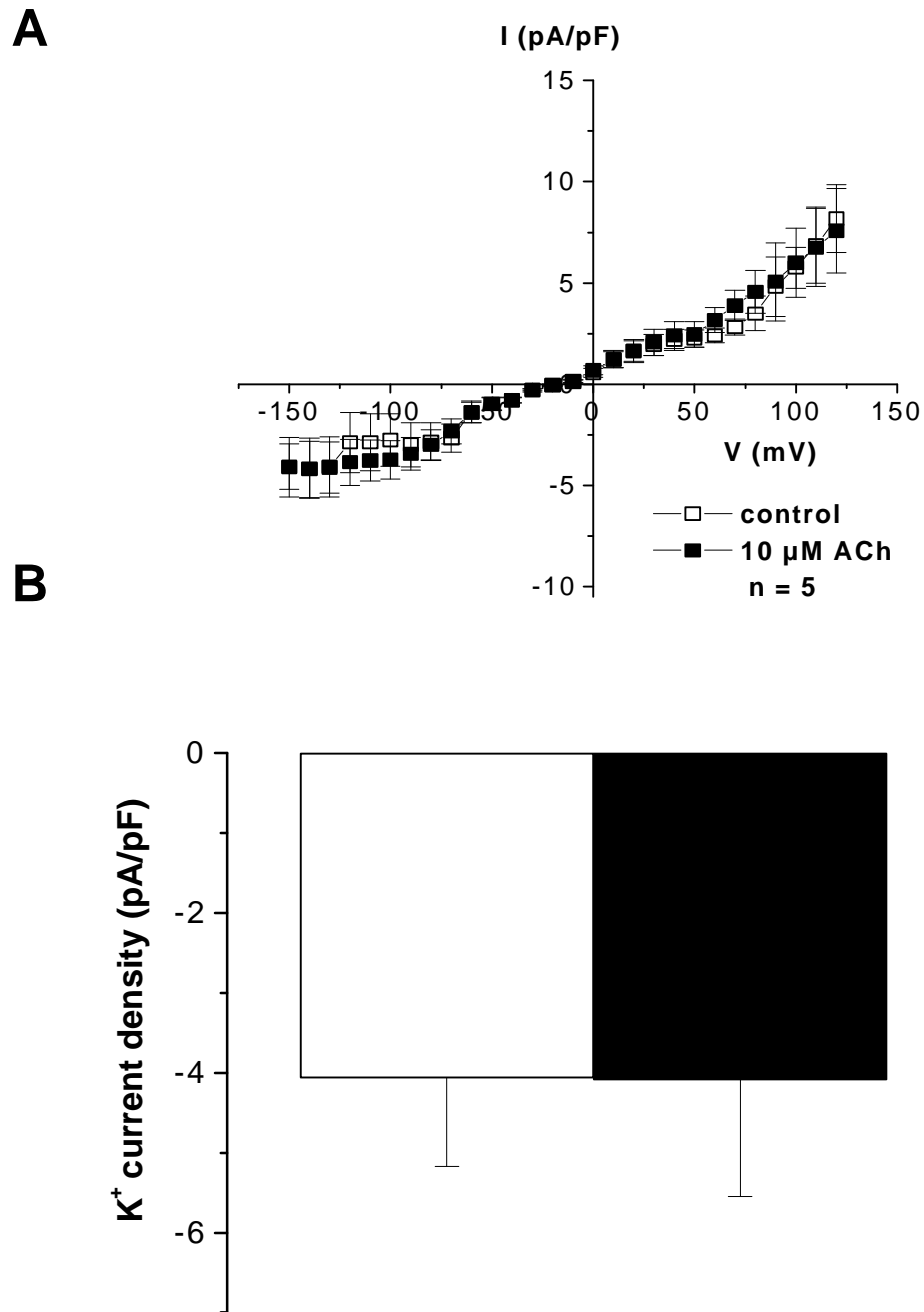


Figure 4.3. Effect of ACh on the background whole-cell K⁺ currents in non-transfected HEK-293 cells. **A.** I-V relationships of background K⁺ channels transmembrane currents before and after the application of ACh at 10 μM. **B.** Summary of the effect of 10 μM ACh on background K⁺ currents (test potential, -150 mV; holding potential, -20 mV).

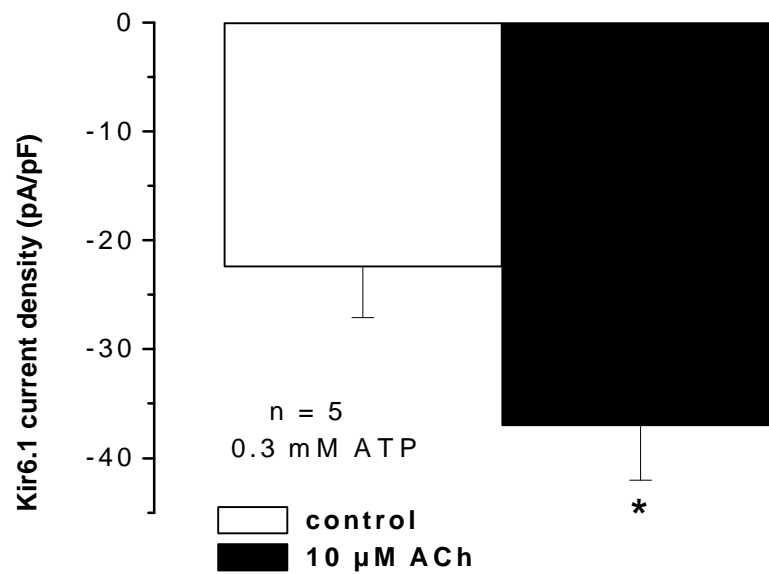
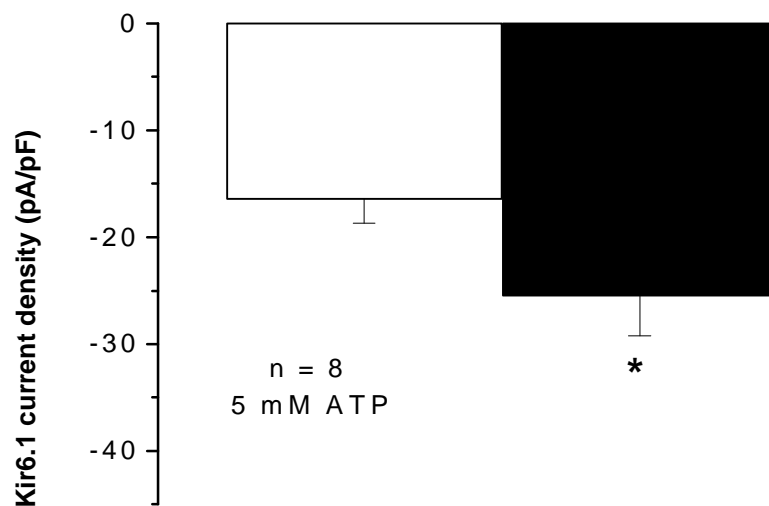
A**B**

Figure 4.4. The stimulatory effects of ACh on heterologously expressed Kir6.1 channels with different concentrations of ATP in the pipette solution. **A.** ACh (10 μ M) increased Kir6.1 currents with 0.3 mM ATP in the pipette solution (n = 5). **B.** Summary of the effect of 10 μ M ACh on Kir6.1 currents (test potential, -150 mV; holding potential, -20 mV) with 5 mM ATP in the pipette solution (n = 8). * p < 0.05 vs. control.

4.3.3. Modification of ACh effect on Kir6.1 channels by different membrane receptors

A general problem in identifying muscarinic receptor subtypes present in a tissue is the lack of subtype selective muscarinic antagonists. Thus, it is necessary to use a combination of various antagonists. Atropine, a nonselective antagonist of mAChR, at 2 μM did not alter the stimulation of Kir6.1 channels by 10 μM ACh (-22.1 ± 4.3 vs. -36.6 ± 3.1 pA/pF, $n = 4$) (Fig. 4.5A and B). To exclude the possible involvement of other mAChRs, we increased the concentration of atropine to 20 μM and 10 μM ACh still stimulated Kir6.1 currents (-17.4 ± 4.2 vs. -31.5 ± 4.7 pA/pF, $n = 4$) (Fig. 4.5C and D). To evaluate the involvement of other receptors in the effects of ACh, we investigated the effect of ACh in the presence of 100 μM mecamylamine (nAChR antagonist), 2 μM prazosin (α_1 adrenoceptor antagonist), and 1 μM propranolol (a non selective β -adrenoceptor blocker) in the bath solution (Wang and Wang, 2000). These different blockers had no influence on the stimulatory effect of ACh on Kir6.1 channels (Fig. 4.6A). α -bungarotoxin (α -BTX), a specific α_1 (Sciamanna et al., 1997) and α_7 (Ji et al., 2002) nAChR antagonist did not block the stimulatory effect of 10 μM ACh (-21.2 ± 4.4 vs. -39.0 ± 4.9 pA/pF, $n = 4$) (Fig. 4.6B). Dihydro- β -erythroidine (D β E) is a competitive antagonist of the human $\alpha_4\beta_2$ nAChRs at micromolar concentrations (Tang et al., 1999). Pretreatment of HEK-293 cells with 10 μM D β E did not alter the stimulatory effect of 10 μM ACh on Kir6.1 currents (-25.8 ± 4.5 vs. -43.4 ± 4.3 pA/pF, $n = 5$) (Fig. 4.7).

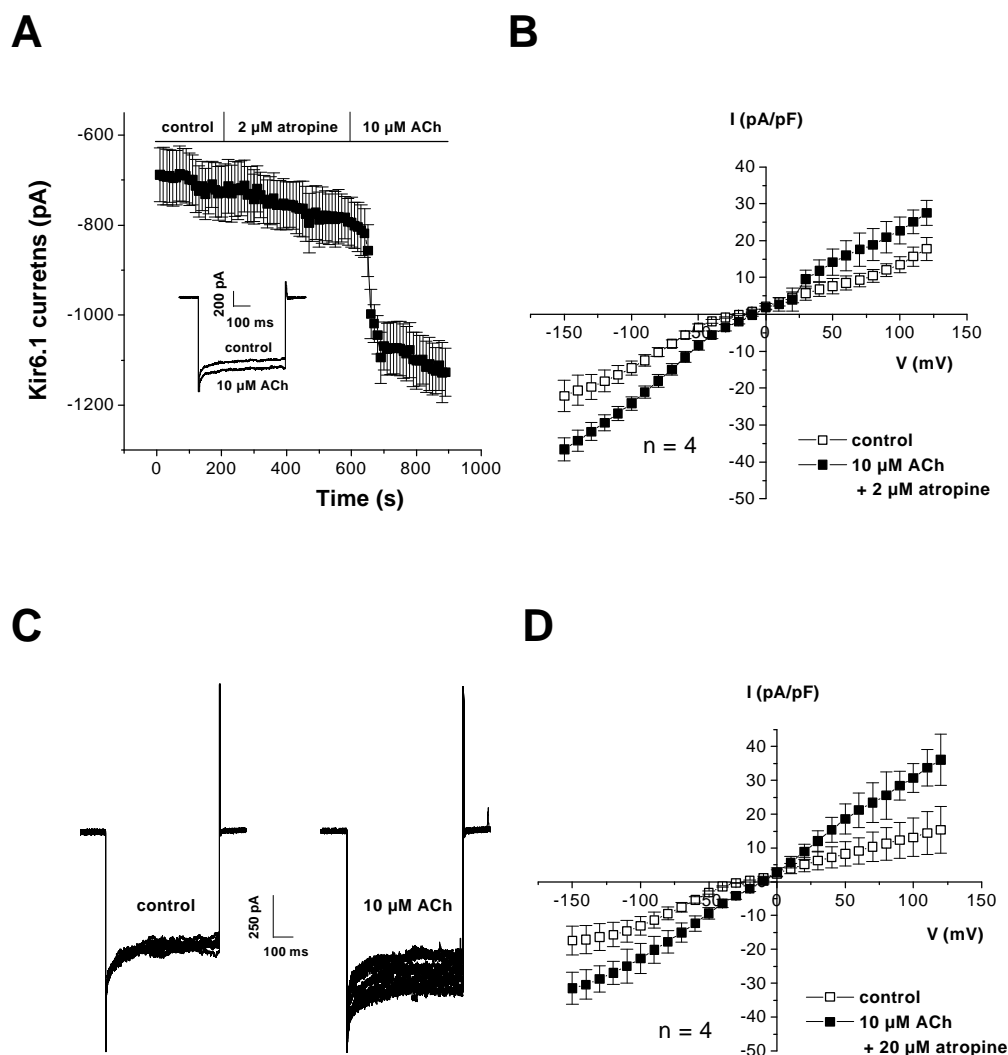


Figure 4.5. The stimulatory effects of ACh on Kir6.1 channels in stably transfected HEK-293 cells. **A.** Time-dependent stimulation of Kir6.1 channels by 10 μ M ACh in the presence of 2 μ M atropine. Test potential -150 mV; Holding potential, -20 mV. **B.** I-V relationship of Kir6.1 channels (n = 4) with 2 μ M atropine in the bath solution. Holding potential, -20 mV. **C.** The original recording of Kir6.1 currents before and after the application of 10 μ M ACh in the presence of 20 μ M atropine in the bath solution. Test potential -150 mV. **D.** I-V relationship of Kir6.1 channels at control and after application of 10 μ M ACh with 20 μ M atropine in the bath solution.

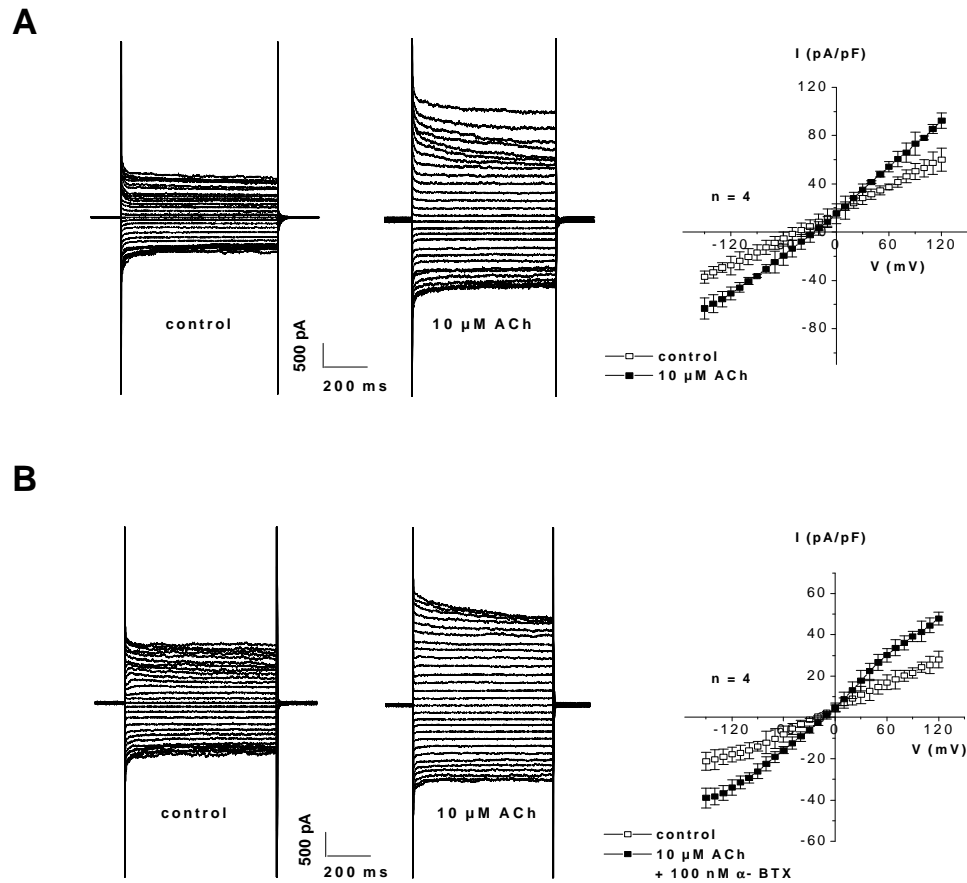


Figure 4.6. The effects of ACh on Kir6.1 channels in the presence of different membrane receptor antagonists. **A.** The original recording of Kir6.1 currents and the I-V relationship of Kir6.1 channels before and after application of 10 μ M ACh in the presence of mecamylamine (100 μ M), prazosin (2 μ M), and propranolol (1 μ M). * $p < 0.05$ vs. control. **B.** The original recording of Kir6.1 currents and the I-V relationship of Kir6.1 channels before and after application of 10 μ M ACh in the presence of 100 nM α -BTX. Holding potential, -20 mV.

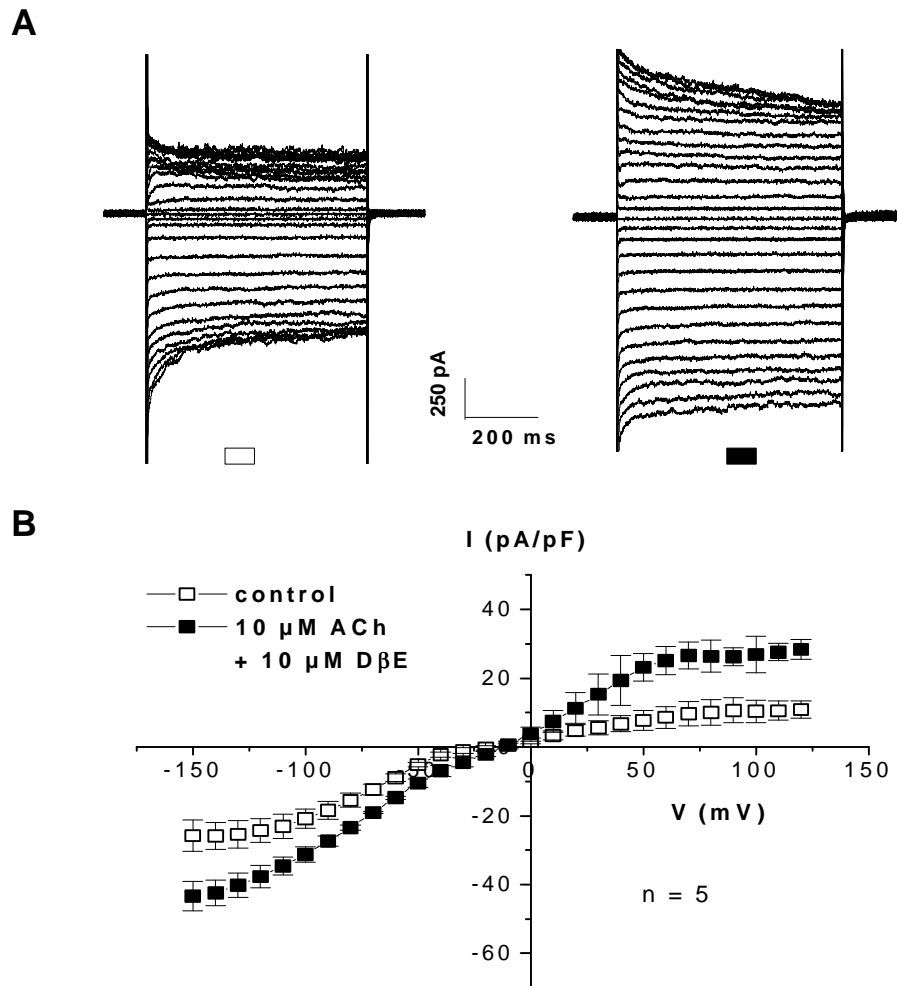


Figure 4.7. The effects of ACh on Kir6.1 channels in the presence of dihydro- β -erythroidine (D β E). **A.** The original recording of Kir6.1 currents. **B.** I-V relationship of Kir6.1 channels before and after the application of 10 μ M ACh with 10 μ M D β E in the bath solution.

4.4. DISCUSSION

We have characterized the stimulatory effect of ACh on Kir6.1 channels stably expressed in HEK-293 cells using the whole cell patch-clamp technique. Our major novel finding is that ACh stimulates Kir6.1 subunits. This effect is not mediated by AChR dependent mechanisms for the stimulatory effect of ACh was not altered by muscarinic or cholinergic receptor blockers.

Northern blot and Western blot analyses have shown the absence of nAChRs in native HEK-293 cells (Chavez-Noriega et al., 2000). Wong et al. (1995) reported that native HEK-293 cells responded to applications of ACh with slowly activating inward currents. The onset of this inward current was delayed approximately 2 seconds following drug delivery. In addition, this current exhibited a prolonged time-course, typically lasting 15 –20 seconds. When HEK-293 cells were challenged with increasing concentrations of ACh, the magnitude of the current increased in a dose-dependent fashion. ACh activated these slow responses with an EC_{50} of $65 \pm 13 \mu\text{M}$ and a Hill coefficient near 1. The slow ACh response was inhibited >90% by 1 μM atropine. This study raised the possibility that HEK-293 cells may possess native mAChRs. On the contrary, the expression of mAChRs in HEK-293 cells at the mRNA level or the protein level has not been reported, neither has receptor binding experiment been done in these cells. mAChRs display both excitatory and inhibitory effects on cholinergic neurotransmission by coupling to different intracellular signal transduction pathways. Whereas the binding of ligands to mAChR subtypes M1, M3 and M5 activates protein kinase C (PKC) and the mitogen-activated protein (MAP) kinase pathway, activation of M2 and M4 mAChRs is known to inhibit adenylate cyclase (Teber et al., 2004). In our

study the effects of ACh on Kir6.1 channels were not altered by mecamylamine (a nAChR antagonist), prazosin (an α 1-adrenoceptor antagonist), propranolol (a β -adrenoceptor blocker), atropine (a muscarinic AChR antagonist), and D β E (a competitive antagonist of human α 4- β 2 nAChRs). These results indicate that ACh stimulation of Kir6.1 channels is most likely the consequence of the interactions between ACh molecules and Kir6.1 channel proteins. Electrostatic and hydrogen bonding interactions between ionized amino acids are known to be important for ion channel subunit interaction and protein folding. ACh is a positively charged molecule with quaternary group of the choline moiety as its positively charged tail (Sussman et al., 1991; Sarri et al., 2004). The pore forming region (H5) of Kir6.1 channel is also positively charged due to two arginine residues (Inagaki et al., 1995). Since ACh molecules cannot pass through cell membranes, it is possible that the positively charged ACh interacts with the positively charged H5 region of Kir6.1 protein, causing conformational changes of the subunit and channel activation. This speculation awaits experimental confirmation.

5. GENERAL DISCUSSION

5.1. PROPERTIES AND CONCENTRATIONS OF NICOTINE USED IN OUR STUDY

Nicotine is the main constituent of tobacco smoke responsible for the elevated risk of cardiovascular disease and sudden coronary death associated with smoking presumably by provoking cardiac arrhythmia. The concentrations of nicotine used in our study ranged from micromolar to millimolar levels. Similar micromolar concentrations of nicotine have been used in other studies. Wang et al. (2000a) studied the effect of nicotine (100 μM) on K^+ channels that were expressed in *Xenopus* oocytes. Uteshev et al. (1996) investigated the effect of 50 μM nicotine on rat hypothalamus neurons. The micromolar concentrations of nicotine used in our study simulate the steady-state plasma levels seen in smokers and smokeless tobacco users. Lin et al. (1992) demonstrated that 6 weeks of nicotine intake through drinking water at a dose of 5 mg/kg/day yielded a mean plasma nicotine level of approximately 1 $\mu\text{g ml}^{-1}$ (2.2 μM). This plasma level of nicotine is approximately equivalent to the intake of nicotine by a 60-kg heavy smoker (40-100 cigarettes per day) (Hui et al., 1997; Lin et al., 1992). A person smoking one cigarette absorbs about 1-1.4 mg nicotine (Feyerabend et al., 1985), leading to a blood nicotine level of 70 nM (Russell and Jarvis, 1985). Nicotine intake from a smokeless tobacco user is even higher. Regular smokeless tobacco users can intake more than 1000 mg nicotine daily (Benowitz et al., 1990), which would lead to a plasma nicotine level of about 60-100 μM . This concentration is comparable to the concentrations used in our present study at the micromolar level. The local concentration of nicotine in different tissues may be even higher, which is especially important for the smokeless

tobacco users. As high as 1 to 10 mM nicotine has been detected in the saliva of snuff users (Hoffman & Adams, 1981). Nicotine at millimolar concentrations seems to be of little biological relevance; however nicotine at 61 mM has been used to characterize the role of neuronal nAChRs in oral irritation and pain (Carstens et al., 2000).

It has been assumed, and to some extent has become public health policy, that it is less hazardous to smoke cigarettes that deliver a lower yield of tar and nicotine than it is to smoke other cigarettes. There is evidence, however, that cigarette smokers regulate their inhalation to maintain nicotine intake. Thus switching from high to low nicotine cigarettes may result in as great or even greater intake of combustion products which are as toxic as or even more toxic than nicotine. The use of nicotine chewing gum has been proposed to assist with smoking cessation. This would be expected to result in lower intakes of combustion products but as much or more nicotine. The use of chewing tobacco and snuff, which deliver considerable amounts of nicotine, is growing. An important consideration in evaluating the risks involved in each of the above cases is the question of the harmfulness of nicotine, which is still largely unknown. Cigarette smoking is known to activate the sympathoadrenal system, increasing heart rate, blood pressure, and circulating levels of catecholamines and corticosteroids (Cryer et al., 1976; Cryer et al., 1977). Most or all of these actions are believed to be mediated by nicotine.

Nicotine is distilled from burning tobacco and is carried proximately on tar droplets and probably also in the vapor phase, which is inhaled. Absorption of nicotine across biological membranes depends on pH. Nicotine is a weak base with a pKa (index of ionic dissociation) of 8.0 (aqueous solution, 25°C). This means that at pH 8.0, 50 % of nicotine is nonionized. In its ionized state, such as in acidic environment, nicotine

does not rapidly cross membranes. Therefore, the pH of tobacco smoke is important in determining absorption of nicotine from different sites within the body. The smoke from air-cured tobacco, the predominant tobacco in pipes, cigars, and in a few European cigarettes, is alkaline with progressive puffs increasing its pH from 6.5 to 7.5 or higher. At alkaline pH, nicotine is largely nonionized and readily crosses membranes. Nicotine from products delivering smoke of alkaline pH is well absorbed through the mouth. When tobacco smoke reaches the small airways and alveoli of the lungs, the nicotine is rapidly absorbed. The rapid absorption of nicotine from cigarette smoke through the lung occurs because of dissolution of nicotine at physiological pH (approximately 7.4), that facilitates transfer across cell membranes. Concentrations of nicotine in blood rise quickly during cigarette smoking and peak at its completion. Armitage and coworkers (1975), measuring exhalation of radiolabeled nicotine, found that four cigarette smokers absorbed 82 to 92% of the nicotine in mainstream smoke, another smoker presumed to be a noninhaler absorbed 29%, and three nonsmokers (who were instructed to smoke as deeply as possible) absorbed 30 to 66 percent.

Chewing tobacco, snuff, and nicotine polacrilex gum are of alkaline pH as a result of tobacco selection and/or buffering with additives by the manufacturer. The alkaline pH facilitates absorption of nicotine through mucous membranes. The rate of nicotine absorption from smokeless tobacco depends on the product and the route of administration. With fine-ground nasal snuff, blood levels of nicotine rise almost as fast as those observed after cigarette smoking. The rate of nicotine absorption with the use of oral snuff and chewing tobacco is more gradual. Nicotine is poorly absorbed from the stomach due to the acidity of gastric fluid, but is well absorbed in the small intestine, which has a more alkaline pH and a large surface area. Bioavailability of nicotine from

the gastrointestinal tract (that is swallowed nicotine) is incomplete because of presystemic metabolism (first pass metabolism), whereby after absorption into the portal venous circulation, nicotine is metabolized by the liver before it reaches the systemic venous circulation. This is in contrast to nicotine absorbed through the lungs or oral/nasal mucosa, which reaches the systemic circulation without first passing through the liver. Nicotine base can be absorbed through the skin. There have been cases of poisoning after skin contact with pesticides containing nicotine. Likewise, there is evidence of cutaneous absorption of and toxicity from nicotine in tobacco field workers.

After absorption into the blood, which is at pH 7.4, approximately 69% of the nicotine is ionized and 31 % nonionized. Binding to plasma proteins is less than 5%. Nicotine is distributed extensively to body tissues with a steady state volume of distribution averaging 180 liters (2.6 times body weight). This means that when nicotine concentrations have fully equilibrated, the amount of nicotine in the body tissues is 2.6 times the amount predicted by the product of blood concentration and body weight. The pattern of tissue uptake cannot be studied in humans, but it has been examined in tissues of rabbits by measuring concentrations of nicotine in various tissues after infusion of nicotine to steady state. Spleen, liver, lungs, and brain have high affinity for nicotine, whereas the affinity of adipose tissue is relatively low.

After rapid intravenous injection (i.v.), concentrations of nicotine decline rapidly because of tissue uptake of the drug. Shortly after i.v. injection, concentrations of nicotine in arterial blood, lung, and brain are high, while concentrations in tissues such as muscle and adipose are low. The consequence of this distribution pattern is that uptake into the brain is rapid, occurring within 1 or 2 min, and blood levels fall because of peripheral tissue uptake for 20 or 30 min after administration. Thereafter, blood

concentrations decline more slowly, as determined by rates of elimination and rates of distribution out of storage tissues.

5.2. NICOTINE-INDUCED PRODUCTION OF SUPEROXIDE ANIONS

In HEK-293 cells stably transfected with the Kir6.1 subunit, nicotine at 100 μ M increased the production of $O_2^{\cdot-}$ while at 1 mM nicotine inhibited $O_2^{\cdot-}$ production. There was a significant difference in the basal level of $O_2^{\cdot-}$ production between the transfected and the non-transfected HEK-293 cells. It is possible that the expression of a heterologous protein (Kir6.1) is causing compensatory changes in the HEK-293 cells leading to the differences in $O_2^{\cdot-}$ production. Further experiments are needed to clarify this point. Initial experiments indicated that non-transfected HEK-293 cells did not exhibit specific [3 H]($-$)-cytisine binding or ($-$)-nicotine-stimulated $^{86}\text{Rb}^+$ efflux. In earlier studies, it has been reported that non-transfected HEK-293 cells had no response after the application of agonists including ACh and nicotine (Gopalakrishnan et al., 1995). In our study the nicotine effect is not receptor mediated. Given the reported lack of nAChRs or mAChRs in HEK-293 cells, we propose that the effect of nicotine is partially due to the production of $O_2^{\cdot-}$ and in another part to the direct effect of nicotine on Kir6.1 channels. In the presence of Kir6.1 Ab in the pipette solution the stimulatory effect of 100 μ M nicotine was abolished in our study. Nicotine at 1 mM exhibited reduced inhibitory effect compared to that achieved in the absence of Kir6.1 Ab in the pipette solution. These results could be explained by two mechanisms. First, Kir6.1 Ab is directed against 79 aa fragment of Kir6.1 protein at its C terminus (Sun et al., 2004). This specific epitope of Kir6.1 may participate in the formation of the K^+ permeation

pore. Therefore when the pore is blocked, nicotine could not exhibit its effects. This led us to the possibility that nicotine and the Kir6.1 Ab may have the same binding site on the channel protein. Second, when Kir6.1 Ab binds to the Kir6.1 channel it may cause conformational changes of the Kir6.1 subunit leading to the change of the configuration of the channel protein and decrease of the opening of the channel. Further studies about the structure of the channels are needed to clarify these possibilities.

In mammalian cells there are two types of K_{ATP} channels. Sarcolemmal K_{ATP} channels and mitochondrial K_{ATP} channels. Sarcolemmal K_{ATP} channels are responsible for setting membrane excitability in response to metabolic stress. Mitochondrial K_{ATP} channels are implicated in the regulation of mitochondrial ion and volume homeostasis. Mitochondrial K_{ATP} channels are the key effectors of cardioprotection. Ischemic preconditioning is an endogenous protective mechanism in which brief periods of myocardial ischemia and reperfusion render the myocardium resistant to a subsequent more sustained ischemic insult (Murry et al., 1986). It has been proposed that in the mitochondria, K^+ channel openers target mitochondrial K_{ATP} channels. This conclusion was based primarily on the observation that the K_{ATP} opener diazoxide produced a cardioprotection effect in isolated rat heart at a concentration which was shown to be selective for opening the mitochondrial, not the sarcolemmal K_{ATP} channel (Grover et al., 1997). In addition, these investigations showed that 5-hydroxydecanoic acid (5-HD), a selective mitochondrial K_{ATP} channel antagonist, blocked the cardioprotective effects of diazoxide lending further support to their hypothesis.

It has been proposed that mitochondria contain mitochondrial K_{ATP} channels and charybdotoxin-sensitive K^+ channels. It has not been established whether the intramitochondrial K^+ concentration is less or greater than the cytoplasmic K^+

concentration, and therefore whether opening of mitochondrial K^+ channels results in potassium influx or release is not very clear. Recent studies have identified candidate mitochondrial K_{ATP} channel proteins, including a 28-kDa protein that was isolated from the heart muscle mitochondria based upon its selective binding to the sulphonylurea compound glibenclamide (Szewczyk et al., 1997), and a charybdotoxin-sensitive channel (Xu et al., 2002). Mitochondrial K_{ATP} channels exhibit a distinct pharmacological profile that allows their functions to be studied; they are selectively activated by low concentrations of diazoxide and blocked by 5-HD (Jaburek et al., 1998; Sato et al., 2000; Liu et al., 2001).

Liu and colleagues (2003) reported a surprising finding that mitochondria apparently release K^+ upon exposure to the mitochondrial K_{ATP} channel opener diazoxide. 5-HD prevented the release of K^+ from mitochondria, consistent with efflux through mitochondrial K_{ATP} channels being a major route of K^+ efflux. The cytosolic K^+ concentration is known to be approximately 145 mM, but the mitochondrial matrix K^+ concentration has not been established due to methodological difficulties. Thus, conflicting data have arisen from different studies (Kowaltowski et al., 2002). Although the assumption has been that the intramitochondrial K^+ concentration is lower than the cytosolic concentration (Debska et al., 2001), the most direct measurements suggest an intramitochondrial K^+ concentration of 180 mM or greater (Garlid, 1996). As we mentioned earlier, nicotine crosses the cell membrane and causes, depending on its concentration, an increase or a decrease of $O_2^{\cdot -}$ production. According to our knowledge there is no reported mechanism of how nicotine could produce its effect on $O_2^{\cdot -}$ production. However, the following mechanisms are proposed (Fig 5.1). Firstly, changes in the activity of mitochondrial K_{ATP} can modulate ROS production and mitochondrial

membrane potential. Membrane potential changes causes cytochrome *c* release and the production of ROS and specifically in our study $O_2^{\cdot-}$. On the other hand, millimolar concentrations of nicotine cause inhibition of mitochondrial K_{ATP} channels and decrease in the activation of the electron transport chain and decrease of ROS production. Secondly, nicotine may trigger mitochondrial membrane permeability changes and the increased production of ROS that may result from mitochondrial membrane alterations. Thirdly, nicotine may effect the Ca^{2+} concentration in the mitochondrial matrix. Ca^{2+} concentration in the cell cytoplasm is 0.1 μM and 1 μM in the mitochondrial matrix. Nicotine may change the inner mitochondrial membrane permeability leading to Ca^{2+} movement to the cytoplasmic space according to its concentration gradient. Ca^{2+} carries two positive charges and its movement outside the mitochondria leads to an increase in the electrical driving force of H^+ ions causing an activation of the electron transport chain and an increase in ROS production. Fourthly, it has been proposed that the mitochondria contain charybdotoxin-sensitive K^+ (K_{Ca}) channels (Xu et al., 2002; Liu et al., 2003). We have previously reported (Tang et al., 1999) that nicotine can modulate K^+ channels. We propose that at micromolar concentration, nicotine activates mitochondrial K_{Ca} channel leading to membrane hyperpolarization and an increase in the production of ROS. Nicotine at millimolar concentrations may cause inhibition of the mitochondrial K_{Ca} channels and mitochondrial membrane depolarization leading to a decrease in ROS production. This decrease in ROS production causes an inhibition of Kir6.1 channels.

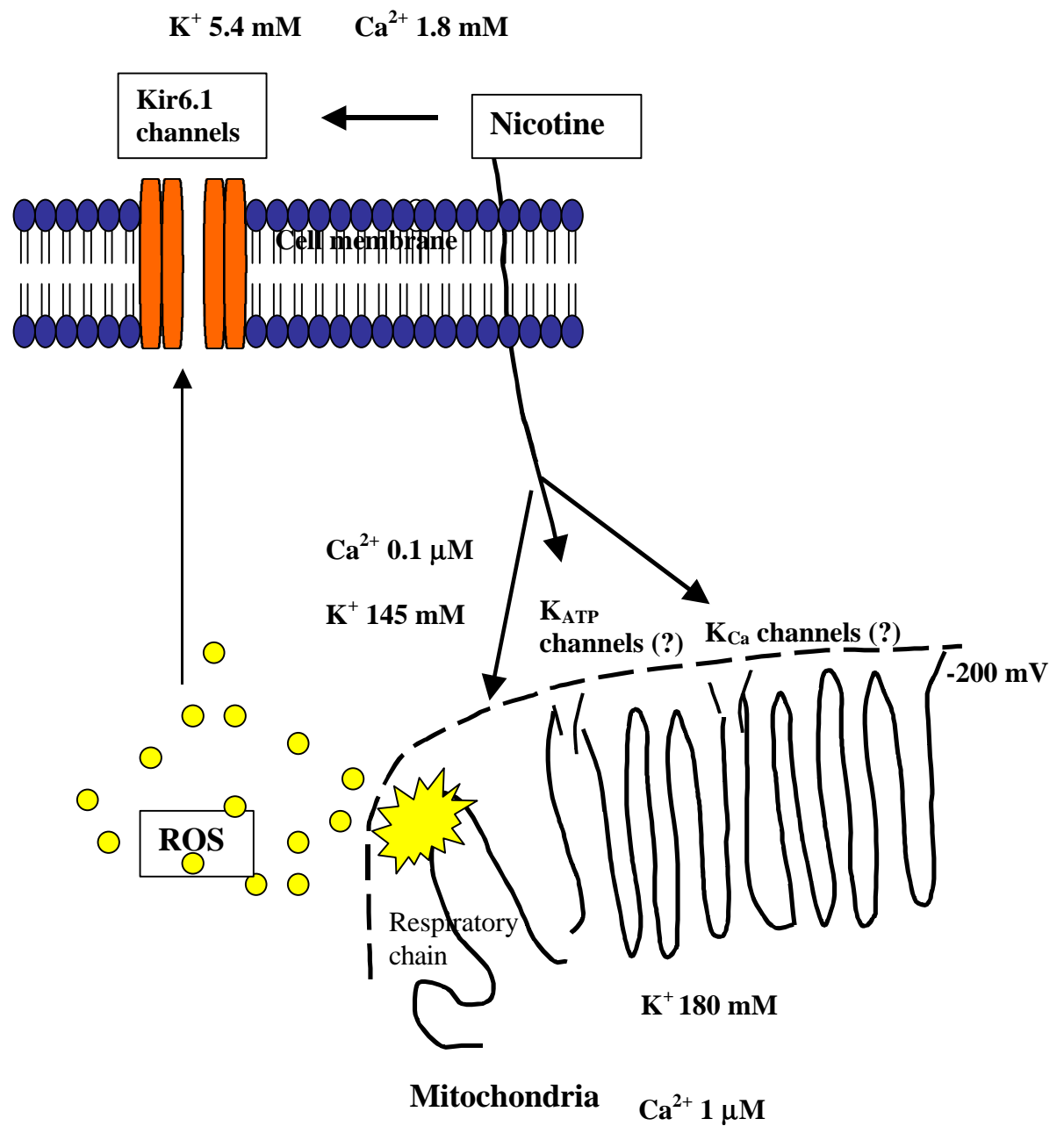


Figure 5.1. The proposed signaling pathway for nicotine. Nicotine causes opening of the mitochondrial K_{ATP}/K_{Ca} channels. The efflux of potassium from the mitochondria causes it to produce reactive oxygen species (ROS) via the electron transport chain.

5.3. MEMBRANE-PERMEABLE RADICAL SCAVENGER TEMPOL

5.3.1. Properties and molecular structure of tempol

Tempol is a free radical scavenger that interferes with the formation or the effects of many radicals, including $O_2^{\cdot -}$, hydroxyl radicals, and peroxynitrite. Tempol is a water-soluble analogue of the spin label tempo, which is widely employed in the electron spin resonance spectroscopy. Tempol is a stable piperidine nitroxide of low molecular weight (molecular weight, 172) that permeates biological membranes (Fig 5.2). Nitroxides are a new class of radioprotectors. Nitroxides have been used as contrast agents in magnetic resonance imaging and as spin labels for biophysical studies. It was the observation that several nitroxides themselves reacted with free radicals, specifically oxyradicals that led to the investigation of these compounds as radioprotectors and antioxidants. McDonald and colleagues (1999) investigated the effects of tempol on 1) the infarct size caused by regional myocardial ischemia and reperfusion of the heart *in vivo* and *in vitro* and 2) the cell injury caused by H_2O_2 in rat cardiac myoblasts. In the anesthetized rat and rabbit, tempol reduced the infarct size caused by regional myocardial ischemia and reperfusion.

5.3.2. Mechanism of action of tempol

Unlike recombinant SOD, which is not able to cross biological membranes, tempol permeates biological membranes and accumulates in the cytosol. There is some controversy as to whether tempol and other stable nitroxides are “SOD-mimetics” or act as stoichiometric scavengers of $O_2^{\cdot -}$. Regardless of its precise mechanism of action, there are many studies that document that tempol attenuates the effects of $O_2^{\cdot -}$ *in vitro*. In

our study we show that the nicotine effect was partially mediated by $O_2^{\cdot-}$ production. The stimulatory effect of nicotine was abolished by 100 μ M tempol. Although the precise mechanism of how tempol could attenuate the effects of nicotine is not clear, we can explain it according to the following studies.

1. Tempol is a water-soluble compound that can permeate biological membranes like the cell membrane. There is a small possibility that tempol could mediate its effects by affecting the membrane fluidity or solubility. Kir6.1 channels are expressed in the plasma membrane thus when membrane fluidity or solubility changes it can change the properties and the opening and closing of the channels expressed. If this is true, then the effect of tempol is mechanical and not due to its effect of scavenging free radicals. However, to our knowledge there are no previous reports or any evidence from our present study to confirm this possibility.

2. It has been suggested that tempol reduces the formation of hydroxyl radicals generated in the Fenton reaction by reducing the intracellular levels of ferrous iron (Monti et al., 1996).

3. McDonald and colleagues (1999) have demonstrated that tempol attenuates the impairment in mitochondrial respiration caused by H_2O_2 in rat cardiac myoblasts in a concentration-dependent manner. The decline in mitochondrial respiration caused by H_2O_2 in these cells is mediated by the generation of hydroxyl radicals. As tempol does not scavenge H_2O_2 , it is likely that tempol prevents the effects of H_2O_2 by preventing the effects of hydroxyl radicals.

4. The hypothesis that many of the beneficial effects of tempol are due to the ability of this agent to scavenge hydroxyl radicals is supported by the following findings. Tempol attenuates the impairment in contractility and reduces the release of lactate

dehydrogenase caused by H_2O_2 in freshly isolated, cardiac myocytes of rat. Tempol also reduced the formation of hydroxyl radicals and the release of lactate dehydrogenase in rat hearts subjected to global myocardial ischemia and reperfusion (Gelvan et al., 1991). McDonald and coworkers (1999) proposed that the protective effects of tempol are due to the ability of this stable nitroxide radical to function as an intracellular scavenger of O_2^- and, particularly, hydroxyl radicals.

5. Nitroxides, such as tempol, can undergo redox reactions to the corresponding hydroxylamine anion or oxammonium cation in biological systems as shown by its ability to react with O_2^- , leading to the formation of H_2O_2 and molecular oxygen. Tempol inhibits the peroxynitrite-mediated nitration of phenolic compounds in the presence of a large molar excess of peroxynitrite, suggesting a catalytic-like mechanism (Carroll et al., 2000).

6. The systemic administration of tempol causes systemic vasodilatation (Zollner et al., 1997). It is likely that this hypotension is at least in part mediated by the metabolism of tempol to hydroxylamine, which is a potent vasodilator. There is also evidence that tempol enhances the bioavailability of NO. Both SOD and tempol increased the signal intensity of the hydroxyl radical adduct by accelerating the dismutation of O_2^- (Zollner et al., 1997).

7. Monti et al. (2001) investigated the mitochondrial effects of tempol in a human promyelocytic leukemic cell line (HL-60). Exposure of these cells to tempol for 24 hrs resulted in a decrease in the intracellular and mitochondrial glutathione pools, an impairment of oxidative phosphorylation, and a decrease in mitochondrial membrane potential. Specifically, tempol affected the function of complex 1 of the respiratory chain.

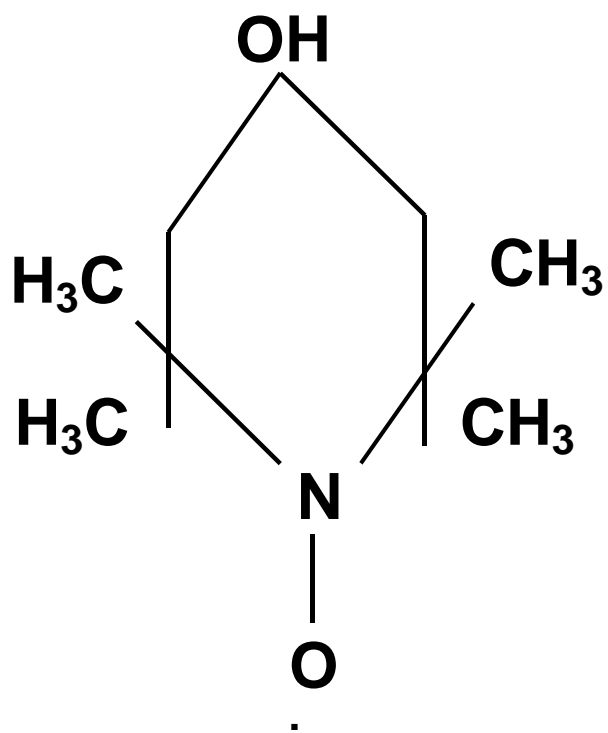
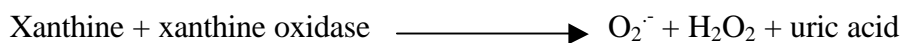


Figure 5.2. Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) is a water-soluble analogue of the spin label tempo.

5.4. HX/XO SYSTEM AND KIR6.1 CHANNELS

In healthy tissue, xanthine oxidase exists as the nicotinamide adenine dinucleotide (NAD)⁺-reducing enzyme called xanthine dehydrogenase. Under normal conditions NAD⁺ accepts electrons without producing any O₂^{•-}. Xanthine dehydrogenase is converted to xanthine oxidase by various mechanisms. During ischemia, it is converted to xanthine oxidase by Ca²⁺-dependent proteases and sulfhydryl group oxidation. During ischemia ATP is catabolized to adenine nucleotides (adenosine diphosphate, adenosine monophosphate, and inosine). Adenine nucleotides are further metabolized to hypoxanthine and xanthine. Biochemical changes during ischemia are the basis of production of ROS.



It is important to mention that the above reactions occur in the body under ischemic conditions. Under our experimental condition there is no ischemia and we have used HX/XO as a source of O₂^{•-} production. We have designed our experiments and took into consideration the factors that might affect our results. Firstly, in order to detect whether there is a signal that is generated from nicotine by itself we included a blank composed of lucigenin, nicotine, and buffer. We observed a very low level of detection in the blank and that value was subtracted from all the data we presented in our study. Secondly, there is the possibility that nicotine might quench the signal of O₂^{•-} production. That nicotine at micromolar concentration significantly increased O₂^{•-} production argues against the quenching of the signal by nicotine. We used a cell-free system to ensure that the system is working. Using only HX/XO without including any

HEK-293 cells demonstrated that there was 7.2 cpm production of $O_2^{\cdot-}$. However, we could have designed another cell-free experiment that includes HX/XO and nicotine. If the $O_2^{\cdot-}$ production was the same in the presence or the absence of nicotine then this would indicate that nicotine has no effect on the signal detection. Thirdly, both the HX/XO system and nicotine produce $O_2^{\cdot-}$. It might be argued that HX/XO might produce such a huge amount of $O_2^{\cdot-}$ that the effect on Kir6.1 channels might be non-specific. HX at 100 μ M and 60 mU of XO produce 7.2 cpm of $O_2^{\cdot-}$. Nicotine (100 μ M) increased the production of $O_2^{\cdot-}$ from 24.6 to 33 cpm/ 10^6 cells. So the actual increase of the $O_2^{\cdot-}$ production is 8.4 cpm/ 10^6 cells. Therefore, the results are valid and there is no excessive production of $O_2^{\cdot-}$ by HX/XO system.

5.5. PROPERTIES OF KIR6.1 ANTIBODIES (KIR6.1 Ab)

Subunit-specific anti- K_{ATP} channel antibodies are important tools for examining the molecular composition and structure-function relationship of native K_{ATP} channels in vascular SMCs. To date, in limited studies anti-Kir6.1 antibodies were produced using either synthetic peptides based on short sequences of Kir6.1 gene or Kir6.1-ST fusion proteins as antigens. None of these antibodies has been used to study the expression of Kir6.1 proteins in the cardiovascular system. Availability of more specific antibodies for the Kir6.1 subunit would greatly facilitate in depth analysis of the expression abundance of K_{ATP} channel proteins and the structure-function relationship of K_{ATP} channels in vascular SMCs. rvKir6.1 cDNA (Genbank No.: AB043637) (rv stands for rat vascular tissue) was isolated from rat mesenteric artery tissues. The cDNA fragment was amplified and manipulated biologically to get a new construct that encodes a GST tag

and an in-frame 79 aa Kir6.1C fragment (L277-A355) (pGEX-Kir6.1C). The recombinant construct was verified by DNA analysis and was sent to the Genbank, and the vascular K_{ATP} channels homogeneity was confirmed. The sensitivity of Kir6.1 Ab antisera was documented as arbitrary absorbance units determined from the chemiluminescence of optical density (OPD). The titer of the Ab was evaluated based on the value of P/N in which P stands for Ab sensitivity and N for OPD absorbance when using bovine serum albumin (BSA) instead as control. The titer of Kir6.1 Ab was as high as 1:1, 280,000 with P/N value ≥ 2.1 . Kir6.1 Ab has very high sensitivity for Kir6.1 channels. The possibility that the Kir6.1 Ab used in our study might interact with ion channels other than the expressed Kir6.1 channels (like Na^+ or Ca^{2+} channels) in HEK-293 is very low for the following reasons. Firstly, non-transfected HEK-293 cells do not contain endogenous Na^+ channels. Therefore these cells are used for stable or transient transfection with cDNA encoding Na^+ channel proteins (Rao et al., 2004; Cummins et al., 2001). Secondly, although Avila et al. (2004) has identified endogenous Ca^{2+} channel currents in HEK-293 cells, there were no Ca^{2+} currents recorded under our experimental conditions. We have included 10 mM EGTA to chelate intracellular Ca^{2+} and inactivate Ca^{2+} channels. In addition, at the testing potential used in our study (-150 mV) most of the Ca^{2+} channels are inactivated. Third, there are no sequence matches with other ion channels. This Ab has very high specificity for K_{ATP} channels and therefore, the possibility that this Kir6.1 Ab might interact with other channels like Ca^{2+} or Na^+ channels is very low.

5.6. SUR AND KIR6.1 SUBUNITS EXPRESSION IN HEK-293 CELLS

Kir6.1 and SUR2B are the constituents of the vascular SMCs. Glibenclamide is a classical sulphonylurea compound that blocks K_{ATP} channels by binding to the SUR subunits. In our study, we used HEK-293 cells as an expression system to express Kir6.1 channels alone. Although this is not a physiological model, our desire was to investigate the effects of nicotine and ACh on Kir6.1 alone. Our future plans are to study the effects of nicotine and ACh on HEK-293 cell co-expressed with SUR2B subunits. We did not co-express SUR subunits in any of the data presented in this thesis. Molecular biological studies from our laboratory showed that, using Western blot analysis that non-transfected HEK-293 cells do not express SUR protein. However RT-PCR studies showed that the SUR subunit was expressed at the mRNA level. These observations mean that although SUR subunit is expressed at mRNA level it can not express a functional ion channel protein. We have demonstrated that Kir6.1 channels were the only subunit expressed in the HEK-293 cells therefore; using glibenclamide as an inhibitor was pointless because it bind to SUR subunits. Instead we used PNU-37883A, which binds to Kir6.1 subunits.

In 1995, several new molecules that were being studied as potential insulinotropic agents for the treatment of non-insulin dependent diabetes mellitus were identified as analogs of meglitinide, previously known as the non-sulphonylurea moiety of glibenclamide. Three of these molecules, namely repaglinide, nateglinide and mitiglinide are or will be soon available for administration to diabetic patients. The binding of the sulphonyureas to SUR1 assumably inhibits the cooperativity of the nucleotide binding folds (NBF)-1 and -2 of SUR1 that induce the closed state of the

K_{ATP} channels. Preclinical studies have shown mitiglinide (KAD-1229), a derivative of benzylsuccinic acid, to have an immediate and short-lasting hypoglycemic action and to increase insulin release from pancreatic β -cells. Mitiglinide has been shown to displace the bound [³H]glibenclamide in the mouse insulin-secreting cell line MIN6 and to inhibit the K_{ATP} channel currents in these cells (Mogami et al., 1994), suggesting that it stimulates insulin secretion by closing the K_{ATP} channels by binding to the sulphonylurea receptors. Repaglinide is a carbamoylbenzoic acid derivative that has been shown to be effective at blocking β -cell K_{ATP} channels and in stimulating insulin release from islets and isolated perfused pancreas. Dabroski et al. (2001) demonstrated that repaglinide blocks recombinant Kir6.2/SUR1, Kir6.2/SUR2A and Kir6.2/SUR2B channels, corresponding to the β -cell, cardiac and SMCs types of K_{ATP} channels, by binding to a site that is located on the SUR subunit. The ability of repaglinide to block all three types of K_{ATP} channels with similar affinity suggests that they share a common binding site.

5.7. NICOTINE AND ITS EFFECTS ON THE CARDIOVASCULAR SYSTEM

Okamura et al. (1993) reported that in the presence of indomethacin, nicotine induced relaxation of the endothelium-denuded dog superficial temporal arterial strips. Wang and Wang (2000) reported that the vasodilatation of rat tail artery rings was observed in the presence of indomethacin. Therefore, the role of prostacyclin (PGI₂) in the nicotine-induced vasodilatation of rat tail artery can be excluded. It is also possible that the nicotine-induced vasodilatation might result from the stimulation of voltage-dependent K⁺ channels in vascular SMCs. In addition to directly inhibiting K⁺ channels,

nicotine has also been shown to significantly increase the amplitude of K^+ channel currents in rat tail artery SMCs via an indirect activation of nicotinic receptors (Tang et al., 1999). To date, no detailed studies on the interaction between nicotine and cloned Kir6.1 channels have been published. The present thesis provides an insight into the potential mechanisms underlying the nicotine effect on Kir6.1 stimulation and vasorelaxation. Mayhan and Sharpe (1998) examined the role of oxygen radicals in nicotine-induced impairment of endothelium-dependent reactivity of resistance arterioles *in vivo*. They reported that acute infusion of nicotine markedly inhibited endothelium-dependent dilatation of resistance arterioles. This finding could not be explained by a non-specific effect of nicotine on vascular reactivity, since responses to nitroglycerine were not altered by infusion of nicotine. In addition they reported that treatment of cheek pouch arterioles with SOD prevented nicotine-induced impairment of endothelium-dependent vasodilatation. The same authors (Mayhan and Sharpe, 1999) reported that chronic injection of nicotine for 2-3 weeks impaired endothelium-dependent, but not endothelium-independent, dilatation of resistance arterioles. In addition, they reported that treatment of the cheek pouch microcirculation with SOD reversed nicotine-induced impairment of endothelium-dependent vasodilatation. The effect of nicotine from cigarette smoke extracts on the alteration of arteriolar dilation *in vivo* in response to stimulation of K_{ATP} channels was studied by Mayhan and Sharpe (1996). High concentrations of nicotine (0.5% and 1%) impaired dilatation of cheek pouch arterioles to activation of K_{ATP} channels and adenylate cyclase. The finding of that study suggests that exposure of resistance arterioles to components of cigarette smoke alters important cellular vasodilator pathways. Many previous studies have examined the effects of activation of K_{ATP} channels on large peripheral and cerebral blood vessels *in vitro* and *in*

vivo. In addition, Mayhan (1993) and others have examined the effects of activation of K_{ATP} channels on peripheral resistance arterioles. In general, activation of K_{ATP} channels with cromakalim, pinacidil, nicorandil, and aprikalim produces marked relaxation and/or dilatation of arteries and arterioles. Relaxation and dilation of arteries and arterioles *in vivo* and *in vitro* appear to be specific because glibenclamide inhibits responses to activation of K_{ATP} channels. Furthermore, Mayhan (1993) has shown that dilation of cheek pouch arterioles in response to activation of K_{ATP} channels with aprikalim and cromakalim is not altered to the synthesis/release of NO or a NO-containing compound. To determine the role of nicotine treatment on reactivity of cheek pouch arterioles to activation of K_{ATP} channels, Mayhan and Sharpe (2002) examined the response to the K_{ATP} channel openers, aprikalim and cromakalim. They reported that acute and chronic exposure to nicotine impaired the dilatation of resistance arterioles to activation of K_{ATP} channels. In addition, they reported that acute treatment of the cheek pouch microcirculation with SOD attenuated the effects of nicotine on dilatation of cheek pouch arterioles in response to activation of K_{ATP} channels. All of the above studies correlated the nicotine-induced impairment of vasodilatation in response to K_{ATP} channel activation to free radical production. Mayhan and Sharpe (2002) reported that treatment of the cheek pouch microcirculation with SOD reversed nicotine-induced impairment of endothelium-dependent vasodilatation. However, none of the above mentioned studies measured the production of $O_2^{\cdot-}$. They reported that nicotine caused impairment of the vasorelaxation induced by K_{ATP} channel activation. Nicotine-induced impairment of relaxation was due to free radical production. SOD treatment reversed the nicotine effect. The second important difference between our studies and Mayhan's study is that their study is *in vivo* study. They measure the diameter of cheek pouch microcirculation

and it was important in that study to keep the endothelium intact. In our study we reported the effect of nicotine on Kir6.1 channels in HEK-293 cells where no endothelium cells are involved. Thirdly, the presence of SUR2B in the cheek pouch arteriole is warranted because as we mention earlier, Kir6.1/SUR2B is the major K_{ATP} channel in vascular SMCs (Cao et al., 2002). The above mentioned studies support our present data in this thesis that nicotine at higher concentrations causes vasoconstriction of the vascular tissue. While in our study we have focused on the expressed Kir6.1 subunits in HEK-293 cells, further experiments are needed to study Kir6.1 coexpressed with the SUR2B subunit to address the effects of nicotine on Kir6.1/SUR2B.

Our data in the present thesis is the first to provide the mechanisms for the effect of nicotine on K_{ATP} channels. Indeed, we have reported here that at micromolar levels nicotine increased the production of $O_2^{\cdot -}$ while at millimolar concentrations it inhibits $O_2^{\cdot -}$ production. Nicotine crosses the cell membrane and causes the production of $O_2^{\cdot -}$ in cytochrome *b* in the electron transport chain in the mitochondria. In the mitochondrion of eukaryotes and in aerobic prokaryotes, cytochrome *b* is a component of respiratory chain complex III - also known as the *bc1* complex or ubiquinol-cytochrome *c* reductase. In plant chloroplasts and cyanobacteria, there is an analogous protein, cytochrome *b6*, a component of the plastoquinone-plastocyanin reductase, also known as the *b6f* complex. Cytochrome *b/b6*, is an integral membrane protein of approximately 400 amino acid residues that probably has 8 transmembrane segments. In plants and cyanobacteria, cytochrome *b6* consists of two subunits encoded by the *petB* and *petD* genes. The sequence of *petB* is colinear with the N-terminal part of mitochondrial cytochrome *b*, while *petD* corresponds to the C-terminal part. Cytochrome *b/b6* non-covalently binds two heme groups, known as *b562* and *b566*. Four conserved histidine

residues are postulated to be the ligands of the iron atoms of these two heme groups. The produced O_2^- causes stimulation of Kir6.1 channels. This is supported by our finding that HX/XO system caused stimulation of Kir6.1 channels when applied to the cells. This may partially explain the stimulatory effect of nicotine on Kir6.1 channels since O_2^- directly stimulated Kir6.1 channels and tempol abolished the stimulatory effect of nicotine on Kir6.1 channels. Another part of the stimulatory effect of nicotine could be mediated by the direct effect of nicotine on Kir6.1 channels. On the other hand, nicotine at millimolar concentrations inhibited the basal O_2^- production. A lower basal level of O_2^- can be linked to a reduced stimulation of Kir6.1 channels. Thus this would underlie the inhibitory effect of nicotine on Kir6.1 channels. The same explanation can be applied to understand the millimolar inhibitory effect on Kir6.1 channels. Nicotine at millimolar concentrations inhibited the production of O_2^- and it even inhibited the basal production of O_2^- , causing inhibition of Kir6.1 currents. Another part of the inhibition may be caused by direct inhibition of Kir6.1 channels.

5.8. EFFECT OF ACh ON KIR6.1 CHANNELS PERMANENTLY EXPRESSED IN HEK-293 CELLS.

In my thesis we investigated the effect of an endogenous neurotransmitter, ACh which shares some characteristics with nicotine, such as the stimulation of ion channels through nicotinic and muscarinic AChRs. Our results clearly show that ACh can stimulate Kir6.1 channels. We hypothesize that the SUR2B subunit may mask the stimulatory effect of ACh on K_{ATP} channels in vascular SMCs. There are two possibilities to explain that hypothesis. Firstly, there are two binding sites for ACh, one

on the Kir6.1 subunit and the other one on the SUR subunit. Binding to those two subunits causes conformational changes in the channel pore leading to blockage of the channel. Secondly, there is only one binding site and it is on Kir6.1 subunit. When the SUR subunit is expressed it causes changes in the three dimensional structure of the channel pore that masks the stimulatory effect of ACh. In order to investigate these hypotheses we need to establish a cell line that co-expresses the Kir6.1 and SUR2B subunit. We would also need to compare that with the effect of ACh on SMCs isolated from mesenteric arteries or aorta because the major constituent of K_{ATP} channels in these tissues is Kir6.1/SUR2B (Cao et al., 2002). On the other hand, SUR2B by itself cannot function as a channel for the lack of the pore forming region in the SUR2B subunits.

Another concern is the relevance of our study to cardiac K_{ATP} channels. As mentioned above, the K_{ATP} channel reconstituted by Kir6.2 and SUR2A exhibits a single channel conductance of ~ 80 pS and is closed by glibenclamide (IC_{50} , ~ 150 nM) and by tolbutamide very weakly (IC_{50} , $\sim > 100$ μ M), and is opened by the KCO pinacidil (EC_{50} , ~ 10 nM). In contrast, diazoxide does not activate Kir6.2/SUR2A channels (Inagaki et al., 1996). All of these characteristics are consistent with those of the K_{ATP} channels recorded in native cardiomyocytes, suggesting that Kir6.2 and SUR2A comprise the cardiomyocyte's K_{ATP} channel. However, several reports suggest the heteromultimerization of Kir6.1 and Kir6.2 subunits within the same channel complex (Kono et al., 2000) while others do not (Seharaseyon et al., 2000). Thus, the molecular structure of the pore-forming subunit of the K_{ATP} channel in native cardiomyocytes has remained controversial. To clarify the molecular structure of the pore subunit of the sarcolemmal K_{ATP} channel in heart, cardiomyocytes from both Kir6.1 null mice (Kir6.1

¹⁻) and Kir6.2 null mice (Kir6.2⁻¹⁻) were examined (Miki et al., 2002). K_{ATP} channel currents (single-channel conductance, ~ 79 pS) were found to be sensitive to ATP and glibenclamide in membrane patches of wild-type but not in Kir6.2⁻¹⁻ cardiomyocytes. In whole-cell membrane recordings, pinacidil produced an outward current that was inhibited by glibenclamide, confirming that it flows through K_{ATP} channels in wild type cardiomyocytes but not in Kir6.2⁻¹⁻ cardiomyocytes. These findings demonstrate directly that Kir6.2 is essential for sarcolemmal K_{ATP} channel function in cardiomyocytes. This was further confirmed by Kir6.2 rescue experiments. In neonatal myocytes of Kir6.2⁻¹⁻ mice, adenoviral gene transfer of Kir6.2 restored the plasma membrane K_{ATP} channel current that was activated by P-1075 and blocked by HMRI 1098, which are a specific activator and a blocker, respectively, of the sarcolemmal K_{ATP} channel (Suzuki et al., 2001). Since the K_{ATP} channel in cardiomyocytes was absent in Kir6.2⁻¹⁻ mice but present in Kir6.1⁻¹⁻ mice, Kir6.2 is an essential constituent of the K_{ATP} channels in cardiomyocytes. The main goal of the present thesis is to study the effect of nicotine and ACh on Kir6.1. Since the main subunit of K_{ATP} channels in the cardiac sarcolemma is Kir6.2, the relevance of our results to cardiac sarcolemmal K_{ATP} channels is weak.

Cardiac myocytes contain two distinct K_{ATP} channels: the classic one in the sarcolemma (Noma, 1983) and another in the mitochondrial inner membrane (mitochondrial K_{ATP} channels) (Inoue et al., 1991). Lethal injury to the heart can be dramatically blunted by brief conditioning periods of ischemia. Such “ischemic preconditioning” effect (Murry et al., 1986) exists in all species examined, including humans (Cohen and Downey, 1993). Although the cardioprotection was originally attributed to sarcolemmal K_{ATP} channels, recent evidence has pinpointed mitochondrial K_{ATP} channels as the key effectors of cardioprotection (Garlid et al., 1997).

Mitochondrial K_{ATP} channels are demonstrated to be composed mainly of Kir6.1/SUR1 by their pharmacological profile. The interaction of Kir6.1 with nicotine and ACh demonstrated in this study may also shed light on the regulation of mitochondrial K_{ATP} channels.

It has been demonstrated that ACh mimicked ischemic preconditioning in anesthetized dogs. Liu and Downey (1993) showed that ACh mimics preconditioning in isolated rabbit hearts. Yao et al. (1999) demonstrated that activation of cardiac mitochondrial K_{ATP} channels increases mitochondrial ROS signaling, thereby activating an important intracellular signaling pathway by which ACh and preconditioning protect against ischemia reperfusion injury. In our present study, neither mitochondrial K_{ATP} channels, nor ischemic preconditioning, which is mainly due to mitochondrial K_{ATP} activation were studied.

In my thesis we propose a new pathway for the modulation of K_{ATP} channels by ACh. There are several important conclusions from our data. First, ACh had no effect on the endogenous K^+ channel currents in HEK-293 cells. Second, ACh stimulated Kir6.1 channels in a dose-dependent manner (Fig. 4.2). Third, the stimulatory effect of ACh was not dependent on ATP level in the cells. The latter conclusion was based on the following two lines of evidence. 1) Intracellular ATP concentration in these studies was clamped at a fixed level (0.3 mM) by dialyzing cells with the pipette solution. 2) Intentionally varying ATP concentrations inside the cells (from 0.3 to 5 mM) did not change the excitatory effect of ACh on K_{ATP} channels although it decreased the basal level of Kir6.1 currents (Fig. 4.4). Fourth, the excitatory effect of ACh on Kir6.1 channels is not mediated by an ACh receptor-dependent mechanism, as had been usually previously believed because the stimulatory effect of ACh is not blocked by atropine.

Fifth, in our study the effects of ACh were not altered by mecamylamine (an nAChR antagonist), prazosin (an α 1-adrenoceptor antagonist), propranolol (a β -adrenoceptor blocker), atropine (a muscarinic AChR antagonists), α - BTX (a specific blocker of α 1 and α 7 nAChRs), and D β E (a competitive antagonist of the human α 4- β 2 nAChRs). These results indicate that ACh stimulation of Kir6.1 currents is most likely the consequence of the interactions between drug molecules and channel proteins.

The effect of ACh on K_{ATP} channels may represent an important endogenous mechanism in SMCs, heart, neurons, and other excitable cells to couple cellular metabolism to excitability. By demonstrating the role of ACh as a novel opener of K_{ATP} channels we begin to understand how the interaction of ACh with Kir6.1 subunits provides an integrated regulation of vascular tone. Kir6.1 subunits are the major component of the mitochondrial K_{ATP} channels therefore our study will provide a better understanding of the ACh effect on cardiac myocytes.

5.9. SUMMARY AND CONCLUSIONS

My thesis presents two important discoveries. First, nicotine, an exogenous substance, at micromolar concentrations stimulated, while at millimolar concentrations inhibited Kir6.1 channels. Nicotine at micromolar levels increased the production of O_2^- while at millimolar level inhibited its production. The effects of nicotine were independent of nicotine receptor stimulation or catecholamine release under our experimental conditions. The nicotine effects are likely partially the consequences of O_2^- production, and partially through interactions between nicotine and Kir6.1 channels. Second, we found that ACh, an endogenous substance released in the body, can dose-dependently activate Kir6.1 channels heterologously expressed in HEK-293 cells. The stimulatory effects of ACh were not blocked by various nicotinic and muscarinic AChR blockers. This is an important new finding because it challenges the traditional well-known pathways of ACh effect through muscarinic and nicotinic AChRs.

Although some synthetic compounds are currently available to open or close K_{ATP} channels for the purpose of experimental or clinical applications, it is still not quite clear where the exact binding sites for these compounds are and whether they are on the Kir6.1 or the SUR subunits. For example, drugs which are used for the treatment of type II diabetes (non insulin dependent diabetes mellitus) earn their importance by their specific binding to SUR2A subunits of K_{ATP} channels in the β -cells of the pancreas. The present study provides evidence that nicotine may be an exogenous and ACh may be an endogenous modulator of Kir6.1 subunits in various tissues. Results derived from this thesis concerning different effects of nicotine and ACh and their mechanisms of action on Kir6.1 channels indicate the importance of the site of action of these two modulators

and have led to more critical evaluation of the mechanism of their actions. This study will also stimulate interest in the identification of other endogenous and exogenous substances responsible for the modulation of Kir6.1 channels.

6. FUTURE DIRECTIONS

To extend and expand our findings reported in this thesis, the following experiments are being developed.

1. Although the effects of nicotine and ACh on K⁺ channels have been studied, no study has investigated the effects of nicotine and ACh on the co-expressed Kir6.1 and SUR2B channels in HEK-293 cells. Under physiological conditions, the Kir6.1 subunit is co-expressed with either SUR1 or SUR2 subunits in native mammalian cells. It is highly possible that in vascular smooth muscle cells Kir6.1 and SUR2B are the major components of the native K_{ATP} channel complex (Cao et al., 2002). It is known that ACh causes vascular tissue relaxation *in vitro* in the presence of endothelium via the production of NO. After the endothelium is removed, ACh does not induce a relaxation or contraction of SMCs. Our results show that ACh stimulated Kir6.1 subunit of K_{ATP} channels. Opening of K_{ATP} channels would cause vasorelaxation. The possible explanation for the discrepancy between our cellular study and the failure of ACh to alter vascular contractility could be ascribed to the presence of the interaction of Kir6.1 and SUR2B subunits in the native vascular SMCs.
2. At least 7 subfamilies are now identified for the inwardly rectifying K⁺ channels designated Kir1.0 - 7.0 (Okuyama et al., 1998). Reconstitution studies have shown that different combinations of Kir6.1 or Kir6.2 and SUR1 or SUR2 variants generate

different K^+ currents. Therefore, whether nicotine and ACh modulate these channels needs to be addressed.

3. Interaction between ROS and K^+ channels has been studied extensively at the pharmacological level. Although significant advances have been made in determining the primary structure of a variety of K^+ channels, our knowledge about the precise sites altered by ROS is still quite limited. Development of specific clones of Kir6.1 channel with mutations at different sites of the amino acid structure, combined with molecular techniques and pharmacological function studies, should help reveal molecular mechanisms by which ROS modulates K_{ATP} channel function. As we mentioned earlier, K_{ATP} are composed of Kir and SUR subunits. It is not clear how ROS interacts with Kir6.x or SUR subunits. Whether other ROS, in addition to superoxide anion, also interact with Kir6.1 is unknown. These questions need to be answered.
4. In the present thesis we used HEK-293 cells as a tool to investigate the effects of nicotine and ACh on Kir6.1 channels. Eventually, we wish to extrapolate our discoveries to the native cells that constitute the cardiovascular system, such as vascular SMCs or cardiac myocytes.

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8. APPENDIX

At the time of printing of this thesis some of its contents have been accepted for publication or published.

Significant parts from chapters 2, 3 and 4 have been submitted for publication in the following journals:

Hanna ST, Cao K, Sun X and Wang R (2004) Modulation of heterologously expressed Kir6.1 channels by nicotine. *Journal of Cardiovascular Pharmacology*, revision.

Hanna ST, Cao K and Wang R (2004) Modulation of heterologously expressed Kir6.1 channels by acetylcholine. *European Journal of Pharmacology*, revision.

Significant parts from chapters 2, 3 and 4 have been published in the following journals:

Ghosh M, Hanna ST, Wang R and McNeill JR (2004) Altered vascular reactivity and K_{ATP} channel currents in vascular smooth muscle cells from Deoxycorticosterone acetate (DOCA)-salt hypertensive rats. *Journal of Cardiovascular Pharmacology*. **44**(5):525-531.

Sun X, Cao K, Yang G, Huang Y, Hanna ST and Wang R (2004) Selective expression of Kir6.1 protein in different vascular and non-vascular tissues. *Biochemical Pharmacology* **67**(1): 147-156.

Hanna ST and Wang R (2002) Effect of calyculin A on the outward K⁺ currents in rat tail artery smooth muscle cells. *J Cardiovascular Pharmacology* **40**: 660-668.